



## Exploring secrets of biofilm and biopolymer production in an engineered system

*Genomic diversity of Zoogloea*

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# EUROBIOFILMS 2024

From environment and basic science  
to serious clinical infections  
- bridging for solutions

Abstract Book

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Wednesday 26 June

## OPENING KEYNOTE

Invited speaker:

### Biofilms in nature and engineered systems: opening the dark box of the huge microbial and exopolymeric diversity

Per Halkjær Nielsen

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Microorganisms growing in biofilms are ubiquitous and crucial in various environmental and engineered ecosystems with an overwhelming part of the microbes uncultured and undescribed. Their importance is not for discussion but beside microbes related to human health, most are very poorly described. How do we get access to the identity, diversity and function of this “dark microbial matter”? Recent technological developments, particular long-read DNA sequencing, open the doors to the microbial genomes, the blueprint for their identity and function. I'll show how this works and give examples of our novel insight in two environments. 1) Biofilms across natural habitats in Denmark, the Microflora Danica project. We have sampled 10,000 habitats across Denmark and are now analysing the diversity, presence of habitat-specific species and their function. The study also allows us to estimate the total number of microbial species in Denmark, of which the vast majority still is very poorly described. 2) Biofilms used for wastewater treatment and resource recovery. We have investigated around 1000 treatment plants across the world where most microbes are novel and where we now are uncovering their identity and function. An important function is their capability to produce extracellular polymeric substances (EPS), important for biofilm formation and treatment plant operation. These EPS are largely unknown and examples of how the composition can be revealed will be given.

Thursday 27 June

## BIOFILMS IN NON-CF CHRONIC RESPIRATORY INFECTIONS

Invited speaker:

### Role of Biofilms in non-CF bronchiectasis: Clinical and lung microbiome impact of chronic versus intermittent *Pseudomonas aeruginosa* Infection in Bronchiectasis

Laia Fernández-Barat 1,2,3\*, Ruben López-Aladid 1,2,3 , Victoria Alcaraz-Serrano 1,2,3 , Nil Vázquez 1,2,3 , Leticia Bueno-Freire 1,2,3 , Roque Pastor-Ibañez 1,2,3 , Lena Lingren 6,7 , Héctor Sanz-Fraile 2 , Patricia Oscanoa 1,2,3 , Ana Motos 1,2,3 , Roberto Cabrera 1,2,3 , Jordi Vila 5 , Daniel Martínez 4 , Jordi Otero 2 , Ramon Farré 2 , Niels Høiby 6,7 , Antoni Torres 1,2,3\*

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4 Pathology Department, Hospital Clínic, Barcelona, Spain;

5 Microbiology Department, Hospital Clínic, CRESIB ISglobal, Barcelona, Spain;

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**Background:** In patients with non- cystic fibrosis bronchiectasis (BE) *Pseudomonas aeruginosa* (PA) has been recently associated with low rather than high number of exacerbations without distinguishing chronic versus intermittent infection. The aim of our study was to determine whether the intermittent or chronic stage of *Pseudomonas aeruginosa* (PA) infection is associated with the rate of exacerbations, quality of life and respiratory microbiome biodiversity after a one-year follow-up.

**Methods:** We conducted a longitudinal study, with 1-year follow-up, in patients with BE intermitendly or chronically infected by PA involving sequential (3-monthly) measurements of microbiological (cultures, PA load, phenotype and biofilms presence) immunological (Serum IgGs against *P.aeruginosa* were measured by ELISA immunoassay) and clinical variables (Quality-of-Life and the number exacerbations). Additionally, 16S sequencing was performed on a MiSeq Platform and compared between chronically infected patients with the mucoid PA versus intermittently infected patients with the non-mucoid PA.

**Results:** We collected 235 sputa and 262 serum samples from 80 BE patients, 61 with chronic and 19 with intermittent PA infection. Chronically compared to intermittently presented reduced quality of life but less hospitalized exacerbations after 1-year follow-up. Chronically infected patients presented reduced sputum biodiversity and higher systemic IgGs against *P.aeruginosa* levels that were associated to decreased number of hospitalized exacerbations.

**Conclusions:** The assessment of Chronic versus intermittent *P.aeruginosa* infection has clinical implications such as quality of life, rate of hospitalized exacerbations and lung microbiome biodiversity. The distinction of these two phenotypes is easy to perform in clinical practice. Screening bronchiectasis patients for the presence or absence of the mucoid *P. aeruginosa* phenotype could provide a better prediction of the burden of chronic *P. aeruginosa* infection within this population. Ultimately, understanding the role of antibodies against *P. aeruginosa* in reducing the occurrence of severe exacerbations warrants further research and could open up new clinical approaches for managing exacerbations.

## Invited speaker:

### Microbiome organization demarcates pulmonary exacerbation types and impacts treatment outcomes in cystic fibrosis.

Stefanie Widder

Multi-species infection of the lung is a hallmark of obstructive lung diseases such as cystic fibrosis (CF), non-CF bronchiectasis, and chronic obstructive pulmonary disease (COPD). The pulmonary microbiota in these conditions typically thrives despite treatment and can hence be considered a dysbiotic microbial system.

Moreover, continued lung infection has been associated with recurring pulmonary exacerbations (PEX) leading to lung function decline and higher mortality rates in these patient populations. An understanding of the microbial underpinnings of PEX is strongly challenged by high inter-patient variability in airway microbial community profiles. Here, we analyzed 880 CF sputum samples organized as near-daily time series using computational modeling and developed non-standard microbiome descriptors to deconstruct community reorganization prior and during 18 PEX events. We identified two communal ecological regimes with opposing organization and dynamics.

Pathogen-governed dysbiosis displayed hierarchical community organization and reduced diversity, whereas anaerobic bloom dysbiosis showed stochasticity and increased diversity. Importantly, we discovered that the background microbiome organization modulated the relevance and activities of focal pathogens and a model simulation of antimicrobial treatment predicted better efficacy for hierarchically organized microbiota. This causal link between PEX, microbiome organization, and treatment success advances the development of personalized dysbiosis management in CF and, potentially, other obstructive lung diseases.

# LESS KNOWN BIOFILM INTERACTIONS

Invited speaker:

## Interaction between oral biofilm and taste receptors

**Xin Zheng**

*D.D.S., Associate Prof., Department of Endodontics, West China School of Stomatology, Sichuan University. Dr. Zheng's long-standing research focus is on the molecular mechanisms of interaction between oral bacteria and host, which exerts a profound impact on dental caries, periodontitis and oral health related systematical comorbidities. His recent findings, published in Nat Commun, Cancer Res, J Dent Res and et al., revealed a bidirectional interaction between taste receptors and oral bacteria, providing a novel target for the management of oral infectious disease.*

The periodontal dysbiosis is the premise for the occurrence and development of periodontitis, and the interaction between oral microbiota and the host is the key to maintaining periodontal homeostasis. Recently, numerous studies reported taste receptors in the airways, gut and urogenital tract were involved in innate immune responses. The current study identified a novel cell cluster named gingival solitary chemosensory cell (gSCC). We found that gSCC express a wide range of T2Rs (bitter taste receptor) that can recognize quorum sensing molecules from oral biofilms, mediate the expression of antimicrobial peptides in periodontal tissues, and regulate the load and composition of periodontal microbiota, which is an important mechanism for maintaining periodontal homeostasis. Subsequent studies found that gingival fibroblasts also express multiple T2Rs, which, when activated, can antagonize LPS-induced NF- $\kappa$ B nuclear translocation and chemokine expression in fibroblast, inhibit neutrophil infiltration, and promote the resolution of periodontal inflammation. These research findings reveal a new mode of microbial-innate immunity interaction in periodontal tissues that is independent of pattern recognition receptors (PRRs), and this interaction mode responds more quickly than the classical mode mediated by PRRs (Figure 1). We conclude that gingival taste receptors may provide a promising target for treating periodontitis by harnessing innate immunity to regulate the oral microbiome. Furthermore, the T2Rs genotype may provide an effective basis for just-in-time screening of periodontitis-susceptible populations.

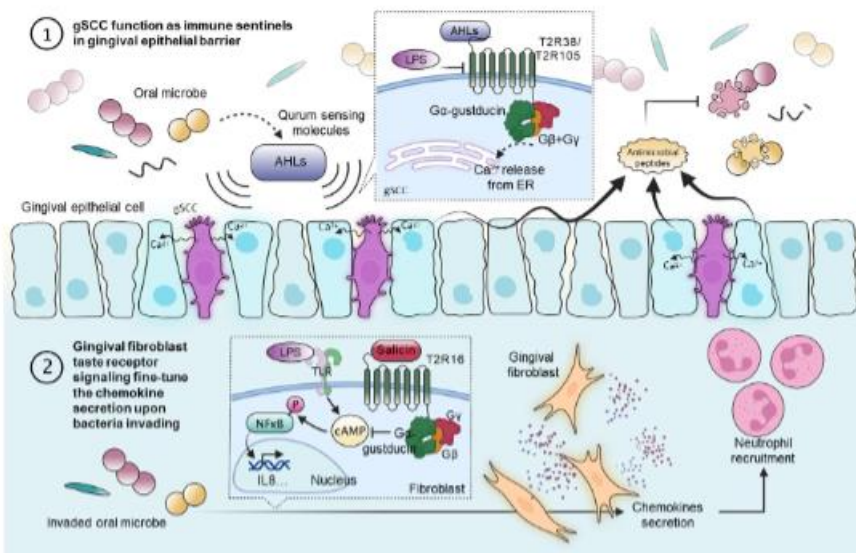


Figure 1. A new mode of microbial-innate immunity interaction mediated by gingival bitter taste receptor signaling.

## Subgingival microbial communities promote epithelial cell migration through regulation of the creatine shuttle

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### Background

The promotion of abnormal epithelial cell migration is one of the important mechanisms by which subgingival microbial communities destroy periodontal tissues and regulate the malignant transformation of epithelial cells. Creatine plays a key role in maintaining adenosine triphosphate (ATP) levels in tissues with high energy demand. Previous studies have confirmed that creatine plays an important role in promoting cell migration. We speculated that creatine plays a role in promoting the migration of subgingival microbial communities. This study investigated the effect of subgingival microbial communities on the creatine shuttle to elucidate the role of creatine in subgingival microbial community-induced cell migration and to explore the potential molecular mechanisms involved in this process.

### Methods

Liquid chromatography was used to determine creatine levels in human oral keratinocyte-1 (HOK-1) cells, human gingival fibroblasts, and human periodontal ligament cells infected with subgingival flora. The expression and methylation of cytosolic vector family 6 member 8 (SLC6A8) were detected using real-time fluorescence quantitative polymerase chain reaction (qPCR), western blotting, reduced representation bisulfite sequencing and methylation-specific PCR. Cell migration and apoptosis were evaluated by Transwell assays, cell healing assays, and flow cytometry. Using transcriptome sequencing, western blotting and RNA interference, the molecular mechanisms underlying how creatine promotes epithelial cell migration were investigated. We also determined the effect of creatine on tissue destruction caused by *Porphyromonas gingivalis* in a mouse model.

### Results

The subgingival microbial community affected the creatine shuttle and ATP levels in a strain-dependent manner. *P. gingivalis*-induced creatine uptake was exacerbated by *Fusobacterium nucleatum* but blocked by *Streptococcus gordonii*. After infection, SLC6A8 expression was upregulated, and SLC6A8 methylation was significantly downregulated. SLC6A8 gene knockdown inhibited creatine shuttling induced by the subgingival flora. Creatine enhances the migration of HOK-1 cells and alveolar bone loss caused by *P. gingivalis* through the protein tyrosine phosphatase receptor F-type peptide interacting protein  $\alpha$ -4 (PPFIA4). SLC6A8 inhibition was also accompanied by a reduction in *P. gingivalis*-induced cell migration.

### Conclusion

Subgingival microbial communities can regulate intraepithelial creatine shuttling, and PPFIA4 can be the target of SLC6A8 for creatine transport. Disruption of SLC6A8 and PPFIA4 expression facilitates *P. gingivalis*-induced increases in epithelial cell migration.

### Keywords:

Microbial community; Creatine; migration; ATP

## Mining Microbial Networks: Deciphering Biofilm-Mediated Horizontal Gene Transfer on Mineral Frontiers

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### Background

Soil, with its diverse surface compositions and characteristics, acts as a magnet for bacterial colonization, fostering the formation of biofilms. These biofilms serve as protective shields against stressors while providing fertile grounds for horizontal gene transfer (HGT), facilitating the exchange of antibiotic-resistant genes (ARG). Our study aimed to elucidate how different soil minerals influence bacterial behavior and to investigate three key aspects: (1) Bacterial survival and membrane integrity, (2) HGT from minerals, and (3) The development of biofilms on minerals.

### Methods

To investigate mineral impact on bacterial behavior: First, we assessed the survival and membrane integrity of two soil bacteria (*Bacillus subtilis* and *Acinetobacter baylyi*) when exposed to different mineral surfaces (goethite, hematite, calcite, kaolinite, quartz, and mica) using CFU/ml assay and LIVE/DEAD staining. This involved subjecting bacterial cells to mineral surfaces with varying structures, charges, and wettability, and then analyzing their viability and membrane integrity. Secondly, we explored the bacteria's ability to uptake plasmid DNA that were pre-adsorbed onto mineral surfaces to simulate HGT events. Lastly, we studied biofilm formation on mineral surfaces using metabolic activity assays, Scanning Electron Microscopy (SEM) to visualize the morphology and Optical Photothermal Infrared (O-PTIR) Spectroscopy for biofilm matrix composition analysis.

### Results

Our findings reveal that mineral surface properties such as structure, charge, and wettability significantly influence bacterial interactions, with highly positively charged nano minerals causing cellular injury. We confirmed that bacteria can acquire DNA from mineral surfaces via HGT, with transformation rates varying based on mineral type. Notably, hydrophilic negatively charged and hydrophobic positively charged minerals exhibited the highest transformation rates, indicating independence from mineral charge and wettability. SEM imaging depicted cluster formation on positively charged surfaces, contrasting with scattered adherences on negatively charged ones. O-PTIR analysis shed light on how substrate properties affect biofilm matrix composition, with higher matrix production intensity observed on positively charged surfaces.

### Conclusion

This research unveils the intricate interplay between minerals, bacteria, and ARG, offering crucial insights into the mechanisms driving ARG dissemination. Such insights hold promise for developing strategies to mitigate gene spread, thereby aiding in the management of antibiotic resistance in natural environments.

### Keywords:

Biofilm, HGT, Soil Minerals, ARG,

Invited speaker:

## Biofilm- related corneal infections by *Pseudomonas aeruginosa*

Liang Yang

Bacterial biofilm formation is well known to contribute to bacterial keratitis which might lead to visual impairment or even loss of the infected eye. *Pseudomonas aeruginosa* is one of the top causative agents of bacterial keratitis. Unlike *P. aeruginosa* lung infections, corneal infections by *P. aeruginosa* still lack mechanistic investigations. *P. aeruginosa* biofilm formation is regulated by the secondary messenger c-di-GMP and the Gac/Rsm two- component regulatory system. In the present study, we first monitored the induction of c-di-GMP signaling during *P. aeruginosa* mouse corneal infection by using the fluorescent c-di-GMP reporter. Next, we performed a comparative transcriptomic analysis to examine the physiology of wild-type *P. aeruginosa* PAO1, a  $\Delta$ wspF mutant strain (high c-di-GMP levels), and a plac-yhjH-containing strain (low c-di-GMP levels) during corneal infections. Those experiments confirmed that biofilm-related genes such as diguanylate cyclase genes and exopolysaccharide genes were induced in *P. aeruginosa* corneal infections. As expected, the  $\Delta$ wspF mutant was less susceptible to host clearance than the plac-yhjH-containing strain and could suppress host immune responses. Our study suggests that elevated intracellular c-di-GMP levels in *P. aeruginosa* are necessary for the establishment of biofilm-associated infection and modulation of host immune responses in mouse cornea.

## The arms-race between amoeba predators and biofilms impacts parasital and bacterial stress tolerance

Ms. Eva Zanditenas<sup>1</sup>, Dr. Liat Rahamim Ben-Navi<sup>2</sup>, Prof. Serge Ankri<sup>1</sup>, Dr. Ilana Kolodkin-Gal<sup>2</sup>

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### Background

In nature, bacterial cells form aggregates in the gastrointestinal tract or structured communities called biofilms that are too large for phagocytosis. Remarkably, trophozoites can specifically recognize and later invade and degrade established biofilms, utilizing a mechanism that mimics digestive exophagy (the secretion of lytic enzymes).

### Methods

We studied the interactions of the human protozoan parasite *Entamoeba histolytica*, an agent causing amebiasis, a disease endemic to developing countries and biofilms formed by enteric bacteria.

### Results

We found that *E. histolytica* cysteine proteinases (CPs) play a crucial role in the degradation process of probiotic *Bacillus subtilis* biofilm. These proteinases target TasA, a significant component of the *B. subtilis* biofilm matrix, also contributing to the adhesion of the parasite to the biofilm. They are also involved in the degradation of biofilms formed by Gram-negative and Gram-positive enteric pathogens and probiotic agents. Our results also indicated that the partial degradation of biofilms protects trophozoites against oxidative stress. These results indicate how protist predators benefit from specifically evolving to distinguish between biofilm and planktonic cells and specifically activate biofilm degradation enzymes (Zanditenas et al., 2023).

Our analysis of the *B. subtilis* biofilm cells released during biofilm predation indicated that the amoeba secretions specifically induced the expression of genes associated with the general stress response. The induction of the Stressosome by amoeba presence allows a subpopulation of cells fleeing from the partially degraded biofilms to tolerate harsh environments and reestablish biofilm communities in undisturbed growth niches.

### Conclusion

Our results indicate that the arms-race between protist predators and biofilms plays a fundamental role in altering stress resistance of microbiome communities and in competitive habitats.

### Reference

Zanditenas, E., Trebicz-Geffen, M., Kolli, D. et al. Digestive exophagy of biofilms by intestinal amoeba and its impact on stress tolerance and cytotoxicity. *npj Biofilms Microbiomes* 9, 77 (2023).

<https://doi.org/10.1038/s41522-023-00444-x>

### Keywords:

Predation; Matrix; Signaling; Stressosome; ROS

## Biofilm Dispersion and Recolonisation by Distinct Phenotypic Lineages

Mr Ciarán Dunne<sup>1</sup>, Ms Dibyangshee Singh<sup>1</sup>, Mr Jacobus Brink<sup>1</sup>, Dr Dishon Hiebner<sup>1</sup>, Professor Eoin Casey<sup>1</sup>

<sup>1</sup>*School of Chemical and Bioprocess Engineering, University College Dublin, Belfield,, Ireland*

### Background

The dispersion of biofilm is associated with the secondary colonisation of surfaces and proliferation of dispersed cells into further biofilm. The localised environment contains different associated phenotypes including biofilm resident cells, dispersed planktonic cells and suspended biofilm aggregates that can be considered distinct phenotypic lineages with varying capabilities for secondary colonisation and development. While biofilm attachment and development are well characterised, a knowledge gap exists regarding dispersion, recolonisation and the dynamics of associated interactions. To determine the influence of dispersed lineages on biofilm development, a methodology was developed for isolating distinct biofilm associated phenotypic lineages before surface inoculation and characterisation.

### Methods

Planktonic cells present in the biofilm vicinity, extracted aggregates of various sizes and extracted biofilm resident cells were isolated from biofilms of *Staphylococcus* and *Pseudomonas* species. Size distributions of the resulting suspensions were determined by dynamic light scattering. Viability was determined through colony forming unit counts and imaging. Confocal laser scanning microscopy was used to quantitatively assess the resulting biofilm. The production and distribution of matrix components by each phenotypic lineage was compared alongside total biofilm biomass, roughness, surface area, attachment efficiency, 3D structure and microcolony distribution. Matrix components were isolated and chemically quantified.

### Results

Distinct biofilm morphological and developmental characteristics may be determined by the historical lineage of resident cells and by dispersed aggregate size. Biofilm-associated planktonic cells showed no associated matrix material and reduced aggregation compared to physically isolated single cells from the biofilm, demonstrating a lower rate of biofilm matrix development and biomass distribution upon secondary colonisation. Isolated biofilm aggregates were diverse in the internal distribution of live and dead cells and extracellular polymeric substances. Biofilm developed from these aggregates demonstrated a size dependent distinction in matrix distribution, biomass development and microcolony morphology.

### Conclusion

Distinct phenotypic differences exist within established biofilms that define the ability of cells or aggregates to recolonise surfaces after dispersion. Dispersal of a given bacterial cell within a biofilm is dependent on localisation and detachment method. This presents important implications for biofilms in water treatment processes and biomedicine as dispersed biofilms can release a range of phenotypes.

### Keywords:

Biofilm, Detachment, Dispersion, Aggregation, Recolonisation

## BIOFILM EVOLUTION AND REGULATION

Invited speaker:

### Evolutionary trajectories of *Klebsiella pneumoniae* biofilm

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*Klebsiella pneumoniae* is recognized as a critically important pathogen widespread globally. Interaction with surfaces, such as urinary catheters, is often a central part of nosocomial *K. pneumoniae* infections. Previously, we have explored how *K. pneumoniae* evolves within-host during a large hospital outbreak. Multiple adaptive changes were selected primarily in urinary tract infections as opposed to gut colonization, which raises a question whether such adaptations are selected due to the presence of an abiotic surface.

We employed an experimental evolution approach where multiple independent lineages of three clinical *K. pneumoniae* strains were subjected to repeated cycles of biofilm growth, dispersal, and reinoculation on catheter-like surfaces. Increased biofilm capacity was rapidly selected via mutations and morphotypes that match those selected clinically. Importantly, there was a substantial genetic parallelism among the strains; however, evolutionary trajectories and resulting phenotypes showed strain differences, illustrating the importance of genetic background on biofilm adaptation. We scrutinized the phenotypes on a cellular, gene expression and biofilm level to show how diverse the properties of evolved mutants are. In connection with survival inside the host, we also assessed other infection-related features, such as survival in human serum, attachment to epithelial cells and acute virulence. Collectively, we found that acute virulence is linked to the underlying genetic change rather than the overall biofilm capacity.

The evolved mutants carried interesting genetic changes not only in the known structures or pathways but also in completely novel factors. In one of the strains, we repeatedly selected a mutation in an uncharacterized S-pyocin domain protein associated with a type VI secretion system that typically functions as an interbacterial weapon. In this peculiar case, a toxic protein mediates a collective behaviour after acquiring a specific point mutation. We showed that the mutant biofilm phenotype is mediated by a fine-tuned ratio between the toxin and immunity proteins which is achieved by increased stability of the toxin with reduced toxicity and altered expression of the immunity-encoding gene. The activity of a less toxic mutant protein induces transcriptional response promoting biofilm phenotype that relies primarily on a c-di-GMP-dependent production of cellulose and type 3 fimbriae.

## Predicting the antibiotic susceptibility of experimentally evolved *Pseudomonas aeruginosa* biofilms using machine learning algorithms

Miss. Fauve Vergauwe<sup>1</sup>, Gaetan De Waele<sup>2</sup>, Prof. Willem Waegeman<sup>2</sup>, Mads Lichtenberg<sup>3</sup>, Prof. Thomas Bjarnsholt<sup>3</sup>, Dr. Callum Highmore<sup>4</sup>, Prof. Jeremy Webb<sup>4</sup>, Margo Cnockaert<sup>5</sup>, Prof. Peter Vandamme<sup>5</sup>, Prof. Filip Van Nieuwerburgh<sup>6</sup>, Prof. Tom Coenye<sup>1</sup>

<sup>1</sup>Laboratory of Pharmaceutical Microbiology, Ghent University, Ghent, Belgium, <sup>2</sup>Department of Data Analysis and Mathematical Modelling, Ghent University, Ghent, Belgium, <sup>3</sup>Costerton Biofilm Center, Department of Immunology and Microbiology, University of Copenhagen, Copenhagen, Denmark, <sup>4</sup>National Biofilms Innovation Centre (NBIC) and Institute for Life Sciences, University of Southampton, Southampton, United Kingdom, <sup>5</sup>Laboratory of Microbiology, Department of Biochemistry and Microbiology, Ghent University, Ghent, Belgium, <sup>6</sup>Laboratory of Pharmaceutical Biotechnology, Faculty of Pharmaceutical Sciences, Ghent University, Ghent, Belgium

Chronic airway infections with *Pseudomonas aeruginosa* are an important cause of morbidity and mortality in patients with cystic fibrosis (CF). Conventional antibiotic susceptibility tests often fail to predict outcome in these patients, mainly because they do not take into account the complex in vivo conditions, and there is a need for alternative approaches to predict antibiotic susceptibility. In the present study we use a combination of experimental evolution, various analytical tools and machine learning approaches to validate a novel approach of predicting *P. aeruginosa* biofilm susceptibility. To this end, *P. aeruginosa* biofilms were experimentally evolved in the synthetic cystic fibrosis medium SCFM2. In this medium, *P. aeruginosa* grows as suspended aggregates, similarly to those observed in CF patients. This was done with six different *P. aeruginosa* strains, either in the presence or absence of tobramycin. Subsequently, the minimal inhibitory concentration (MIC) and biofilm prevention concentration (BPC) were determined and whole-genome sequencing (WGS) data were collected from samples taken at the beginning and end of the evolution experiment. In addition, all strains were characterized by isothermal microcalorimetry (yielding insights into metabolic activity), MALDI-TOF mass spectrometry and multi-excitation Raman spectroscopy.

Combining these different analytical procedures with machine learning, it is possible to link changes in various -omes to different MIC values. To this end, ordinal regression models were trained on each of the different data sources (WGS, microcalorimetry, MALDI-TOF, and Raman spectroscopy) to predict the susceptibility of our evolved isolates. The accuracy of the MIC predictions ranged from 86.96% (Raman spectroscopy and WGS) to 93.48% (MALDI-TOF and microcalorimetry). Our data show the potential of using alternative and faster measures for predicting the antibiotic susceptibility of bacteria.

### Keywords:

biofilms, *Pseudomonas-aeruginosa*, machine-learning, susceptibility-testing

## The Legionella collagen-like protein: a mediator of host-adhesion and biofilm formation

Dr Leanne Cleaver<sup>1</sup>, Dr Saima Rehman<sup>1</sup>, Ms Maria Baczynska<sup>1</sup>, Ms Anna Antonovic<sup>2</sup>, Dr Mark Freeley<sup>2</sup>, Mr Ian McIntire<sup>3</sup>, Mr Huaixin Zheng<sup>3</sup>, Dr Chris White<sup>2</sup>, Mr Carlton Adams<sup>3</sup>, Mr Matteo Palmer<sup>3</sup>, Dr Arianna Fornili<sup>2</sup>, Dr Chris Lorenz<sup>4</sup>, Dr Nicholas Cianciotto<sup>3</sup>, Dr James Garnett<sup>1</sup>

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### Background

Legionella pneumophila (Lp) is a Gram-negative bacterium that can cause severe pneumonia in susceptible individuals. It is found naturally in the environment and in man-made water systems and is transmitted through aerosolised water droplets. The Legionella collagen-like protein (Lcl) is displayed on the surface of Lp and is required for host-adhesion and biofilm formation, which is regulated through divalent cations. We recently determined the structure of Lcl and uncovered a novel mechanism for binding host-glycosaminoglycans (GAGs). The aim of this study was to determine how Mg<sup>2+</sup> can regulate the function of Lcl, how it enables biofilm formation, and whether it has a role in multi-species biofilms.

### Methods

Atomic force microscopy (AFM) was used to assess aggregation of recombinant Lcl, and NMR/X-ray crystallography to assess Mg<sup>2+</sup>-binding to the Lcl C-terminal domain (CTD). A sedimentation assay was used to assess aggregation of Lp strain 130b in the presence of chondroitin-4-sulphate (C4S), purified Lp LPS, and/or MgCl<sub>2</sub>. A pulldown assay was also used to assess binding of the CTD to purified LPS from Escherichia coli, Klebsiella pneumoniae and Pseudomonas aeruginosa.

### Results

AFM showed Lcl does not aggregate in the presence of Mg<sup>2+</sup> while NMR and crystallography showed Mg<sup>2+</sup> binds to the CTD and causes conformational changes. Sedimentation assays showed that MgCl<sub>2</sub> and Lp LPS facilitated aggregation, whereas C4S caused aggregation only at high concentrations. C4S abrogated MgCl<sub>2</sub>- and LPS-dependent aggregation, and the former was inhibited with LPS antibody. The pulldown assay with recombinant CTD demonstrated binding to K. pneumoniae and P. aeruginosa LPS, but not E. coli LPS.

### Conclusion

We have shown that Mg<sup>2+</sup> binds to the CTD of Lcl and causes a conformational change. In this state Lcl can recognise LPS on adjacent bacteria, resulting in bacterial aggregation. Lcl can also bind other bacterial LPS and may suggest a new role in facilitating multispecies growth. We propose that Lcl functions as a molecular switch that modulates between host-adhesion and biofilm formation in Lp, and as we have now identified the Lcl gene outside of Legionella, this may represent a generic mechanism used by a wide range of other Gram-negative bacteria.

### Keywords:

Lcl, Legionella, biofilm adhesin, lipopolysaccharide

Invited speaker:

## Surface colonization of a human pathogen revisited

Urs Jenal

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The ability to efficiently colonize mucosal surfaces is a key requirement for opportunistic pathogens like *Pseudomonas aeruginosa*. But how pathogens collectively respond to surfaces and how individual cells functionally adapt to optimize adherence, virulence, and dispersal is still largely unclear. *P. aeruginosa* optimizes the initial steps of surface colonization by combining tactile sensing with a surface-specific asymmetric virulence program. Surface encounter rapidly boosts the concentration of the second messenger c-di-GMP, which induces tissue adherence and virulence induction by stimulating type IV pili assembly. Surface-attached *P. aeruginosa* cells divide asymmetrically to generate daughters with differential levels of c-di-GMP and different functionalities, a surface-adherent offspring, and a motile progeny that leaves the site of attachment to colonize distant sites. Growth and surface dispersal of *P. aeruginosa* is further promoted by the bimodal expression of HecE, a small protein which boosts c-di-GMP in a subpopulation of cells by modulating the activity of major components of c-di-GMP regulation in *P. aeruginosa*. Our work demonstrates how deterministic and stochastic events determine *P. aeruginosa* cell behavior and how this contributes to efficient surface colonization and biofilm formation.

## Insights into *Vibrio cholerae* Biofilm Formation on Microplastics - the Impact of Quorum Sensing and Plastic Additives

Dr. Kerstin Thriene<sup>1,5</sup>, Dr. Ying Huang<sup>2,5</sup>, Dr. Rupali Sathe<sup>2,5</sup>, Dr. Zoltan Cseresnyés<sup>3</sup>, Prof. Marc Figge<sup>1,3,5</sup>, Prof. Ute Hellmich<sup>4,5</sup>, Prof. Pierre Stallforth<sup>2,5</sup>, Prof. Kai Papenfort<sup>1,5</sup>

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Plastic waste is ubiquitous in ecosystems and poses an increasing ecological challenge with unpredictable consequences. Microplastics, i.e. particles smaller than 5 mm, serve as a surface for microorganisms to form biofilms, the so-called "plastisphere", which favors certain species and can thus disturb the ecological balance. Of particular concern is the colonization of microplastics by pathogenic bacteria such as *Vibrio cholerae*, which can cause severe cholera infections in humans. This poses a clinical threat as these bacteria-carrying microplastic particles (MP) can be ingested by marine organisms and enter the food chain, and the biofilm associated bacteria additionally display increased antibiotic resistance. We therefore aimed to understand the mechanisms underlying the biofilm formation of *V. cholerae* on microplastics to identify potential intervention targets. Therefore, we employed confocal microscopy with image-based quantification and droplet digital PCR to visualize and quantify *V. cholerae* biofilms forming on MP as well as a microfluidic model for live monitoring of biofilm formation and investigation of factors impacting biofilm integrity. Our results show a robust formation of biofilms by *V. cholerae* on MP. Considering the central role of quorum sensing (QS) in biofilm formation, we investigated the effects of the QS molecule DPO on the formation of these microplastic associated biofilms. Here, we observed that wild-type *V. cholerae* was unable to attach to MP in the presence of DPO, whereas mutants lacking the DPO receptor VqmA were able to form structurally normal biofilms. Further, we investigated the effects of chemical additives such as bisphenol A, which are leaching from plastics due to their continuous degradation in the environment, on MP-associated *V. cholerae* biofilms. Our results demonstrate that bisphenols inhibit bacterial growth and trigger *V. cholerae* to form thicker and more enduring biofilms on MP. Applying transcriptome analysis, we were able to identify a distinct response of *V. cholerae* to the presence of bisphenols. Our study demonstrates that *V. cholerae* forms strong biofilms on microplastic particles that are dependent on QS. Furthermore, our results suggest that plastic additives may be a key contributor to the negative impacts of biofilms associated with microplastics.

### Keywords:

*Vibrio cholerae*, biofilm, microplastics

## Uncovering the role of second messenger signalling in *Acinetobacter baumannii* virulence

Miss. Lyuboslava Harkova<sup>1</sup>, Dr Rubén de Dios<sup>1</sup>, Dr Ronan McCarthy<sup>1</sup>

*1Brunel University London, Uxbridge, United Kingdom*

### Background

Second messengers signalling systems play crucial role in bacterial capability to respond to environmental changes by mediating alternations in transcription, translation and enzyme activity. Second messengers are central regulators of various aspects of bacterial life including metabolism, pathogenicity, sessility and cell morphology. Indeed, these molecules are widely recognised mediators of virulence and antibiotic tolerance in well-studied pathogens like *Pseudomonas aeruginosa*. The multidrug resistant nosocomial pathogen *Acinetobacter baumannii* has become increasingly prevalent over the last 20 years with carbapenem-resistant strains surpassing *P. aeruginosa* to become the World Health Organisation top priority pathogen. Despite this, relatively little is known about the role of second messengers in *A. baumannii* virulence and information about the cAMP signalling cascade is scarce, so we aimed to investigate this regulation.

### Methods

In this work, we used high-throughput screening of the *A. baumannii* AB5075 transposon mutant library to identify novel regulators of biofilm formation. A clean deletion mutant of the candidate gene was created and dRNA-seq was used to analyse the transcriptome. Subsequently, the transcriptomic results were phenotypically validated.

### Results

Through the screening to uncover novel biofilm formation regulators, we identified a previously uncharacterised predicted adenylate cyclase (AC), CavA, as a central regulator in *A. baumannii*. We confirmed that CavA AC is functional and synthesizes cAMP. To uncover the CavA-mediated cAMP regulon, we performed a dRNA-seq. This showed that cAMP both upregulated the type IV pili while downregulating Csu pili and exopolysaccharide production gene transcription. We demonstrated for the first time that, cAMP is atop of a hierarchical signalling cascade controlling inter- and intrabacterial signalling by modulating quorum sensing and c-di-GMP systems at transcriptional level, ultimately governing virulence in vivo and adaptive antibiotic resistance in *A. baumannii*. Moreover, we challenge the established norm of high c-di-GMP leading to increased biofilm formation, as our results show that this paradigm is dependent on intracellular cAMP concentrations in this pathogen.

### Conclusions

Overall, we demonstrate the central role of CavA and cAMP in modulating key phenotypes linked to virulence and antimicrobials resistance of *A. baumannii*. This highlights the cAMP signalling cascade as a potential target for novel therapeutic development.

### Keywords:

second messengers, virulence, *Acinetobacter baumannii*

## WHAT'S NEW IN IMPLANT RELATED BIOFILM INFECTIONS?

Invited speaker:

### Implant related biofilm infections

Marjan Wouthuyzen-Bakker

In this lecture, the focus will be on periprosthetic joint infections as example of an implant related infection. Those aspects important to clinicians will be addressed concerning clinical presentation, diagnosis and antibiotic principles in terms of treatment. The aim of this lecture is to build a bridge between fundamental researchers and clinicians in the challenges we are facing in biofilm related infections.

## Bone prosthesis environment influences *Cutibacterium acnes* biofilm formation and composition.

Jennifer Varin-simon<sup>1</sup>, Christine Guillaume<sup>1</sup>, Dr Marius Colin<sup>1</sup>, Dr Frederic Velard<sup>1</sup>, Dr Renaud Siboni<sup>1,2</sup>, Pr Xavier Ohl<sup>1,2</sup>, Dr Celine Mongaret<sup>1</sup>, Pr Sophie Gangloff<sup>1</sup>, Dr Fany Reffuveille<sup>1</sup>

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In the context of the 10% prosthesis joint infections (PJIs) involving *Cutibacterium acnes*, physiopathologic mechanisms are poorly known, whereas a better comprehension of biofilm development leading to PJIs is necessary to help prevent these infections. The bone prosthesis environment, such as type of surface prosthesis or the bone cell secretions, influences biofilm formation. Moreover, other mechanisms influencing bacteria behavior as internalization of *C. acnes* in osteoblasts, could have consequence on biofilm.

The aim of this study is to characterize *C. acnes* biofilm in bone prosthesis context to better decipher PJIs. The biofilms of two PJI (PJI2 and PJI8) and two non-PJI-related (C2 and C5) strains on plastic and titanium supports and bone explants were characterized by (i) counting adherent and live bacteria, (ii) biofilm matrix fluorescent labeling and (iii) quantification of mRNA expression of genes involved in biofilm formation.

*C. acnes* biofilm characteristics were strain- and support-dependent. Indeed, all 4 strains adhered more on titanium support than on plastic (1.4 to 11.2-fold up). For PJI-related strains, the biofilm matrix analyses revealed higher proteins (7.8 to 11.5-fold up) and polysaccharides (1.7 to 12.4-fold up) signal on titanium compared to plastic. Genes involved in biofilm formation were lower expressed on titanium than on plastic for C2. Conversely an increase of gene expression was observed for C5 on titanium compared to plastic. For PJI-related strains, no real difference was noted.

With regard to titanium, internalization of C2 in osteoblast led to a decrease of adhesion (80.2 fold-down) but an increase of polysaccharides fluorescent signal (2.9 fold-up) and an overexpression of all genes involved in biofilm.

Finally, all 4 strains can adhere on bone explant. However, only C2 adhered bacteria were more numerous on fresh bone (containing bone cells) than on frozen bone (after cells lysis) (3.9-fold). To conclude, these results showed that *C. acnes* is able to adapt its biofilm formation and composition according to the microenvironment. *C. acnes* were, strain-dependent, influenced by the nature of the materials but also by internalization in osteoblasts.

### **Keywords:**

*Cutibacterium acnes*, biofilm, prosthesis, internalization

## Bacterial Micro-Aggregates as a Clinically Relevant Inoculum in a Pig Model of Implant-Associated Osteomyelitis

Miss. Katrine Top Hartmann<sup>1</sup>, Regitze Lund Nielsen<sup>2</sup>, Freja Cecilie Mikkelsen<sup>1</sup>, Hanne Ingmer<sup>1</sup>, Lasse Andersson Kvich<sup>2</sup>, Bent Aalbæk<sup>1</sup>, Anders Odgaard<sup>3</sup>, Henrik Elvang Jensen<sup>1</sup>, Mads Lichtenberg<sup>2</sup>, Thomas Bjarnsholt<sup>2,4</sup>, Louise Kruse Jensen<sup>1</sup>

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### Background

Existing animal models of implant-related infections are inoculated with freshly grown planktonic bacteria or already well-established surface-attached biofilms. However, neither of these resembles the clinical condition in humans, where bacterial micro-aggregates from the skin are the cause of the implant infection. This study aimed to create a more realistic inoculum of bacterial micro-aggregates, which were used as inoculum in a pig model of implant-associated osteomyelitis (IAO) and compared to a planktonic inoculum.

### Methods

Bacterial micro-aggregates of the porcine *Staphylococcus aureus* strain SP54F9 were grown in Tryptone Soya Broth for seven days. Afterwards, the suspension was filtered through cell strainers of different pore sizes to separate the micro-aggregates and remove most of the planktonic bacteria. The size-separated micro-aggregates were subsequently characterized by light microscopy to evaluate their size, and isothermal microcalorimetry was used to assess metabolic activity. The micro-aggregate fraction of 5-15  $\mu\text{m}$  was tested as inoculum in a pig model of IAO.

Six adult female minipigs were allocated into three groups (n=2) receiving different inoculums: Group A: Planktonic inoculum (*S. aureus* strain SP54F9 from Lysogeny Broth overnight culture, 10<sup>4</sup> CFU in 10  $\mu\text{L}$ ). Group B: 5-15  $\mu\text{m}$  micro-aggregate inoculum (10<sup>4</sup> CFU in 10  $\mu\text{L}$ ). Group C: Control group (sterile 0.9 % saline in 10  $\mu\text{L}$ ).

### Results

The filtration method separated the bacterial micro-aggregates by size but did not eliminate planktonic bacteria. The micro-aggregates presented with a delayed time-to-peak in the isothermal microcalorimetry i.e., showing a lower metabolic activity compared to the planktonic bacteria. Bacteria were re-isolated from soft tissue, bone, and implants from the IAO pig model inoculated with both planktonic bacteria and the micro-aggregates. However, pigs inoculated with micro-aggregates showed less severe lesions together with an altered bone healing response with pronounced osteoid formation comparable to the control group.

### Conclusions

Micro-aggregates of bacteria with a reduced metabolic activity were created by separation into different size fractions, and used as inoculum in a pig model of IAO. The micro-aggregates caused a less aggressive IAO and a more pronounced bone healing response than the standard planktonic inoculum. These micro-aggregates could offer a clinically relevant inoculum for low-grade, slow-onset infection models.

### Keywords:

Micro-aggregates, implant-related infections, animal models

## Invited speaker:

### Novel antimicrobial agents for implant-associated infections

Rikke Louise Meyer 1, Co-authors: Magnus Schou-Jørgensen 2 , Maiken Petersen 1 , Alif Firdausy 1 , Anne Tvillum 2 , Lærke Glud 1 , Mikkel Illemann Johansen 3 , Nis Pedersen Jørgensen 3 , Lars J. Østergaard 3 , and Alexander Zelikin 1

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Several features of biofilms make standard antibiotic therapy ineffective: Some drugs do not penetrate the biofilm well, and those that do are not effective against non-growing persister cells residing in the biofilm. New antimicrobial therapies can therefore benefit from using drug delivery systems to concentrate, release, or activate the drug at the site of infection. To achieve this, we draw inspiration for cancer therapy, where targeted drug delivery has been developed successfully. Some of the drugs used in cancer therapy are also effective against bacterial persister cells. The aim of our research is to repurpose these drugs for treating biofilm infections. We used mitomycin C as a test case for this concept, and developed a targeted drug delivery platform where the drug is conjugated to a targeting molecule (e.g. an antibody) via a biodegradable linker.

We developed mitomycin-antibody conjugates that bind to the surface of *S. aureus*, and we show that the linker molecule is degraded upon contact with the bacterial cell, resulting in drug release and cell death. The antibody-drug conjugates were highly effective against non-growing bacteria and biofilm in vitro and a murine implant-associated bone infection in vivo. Our new strategy is to replace the antibody with vancomycin as a targeting molecule. Vancomycin is better suited for scalable production, has a longer shelf life, and binds Gram positive bacteria broadly. Even though non-growing cells do not incorporate as much vancomycin into the peptidoglycan as growing cells do, the ongoing remodeling of the cell wall is sufficient to bind enough vancomycin-mitomycin conjugates to kill a substantial part of the bacterial population after release of mitomycin from the conjugate. This strategy may open the door to using more potent and toxic drugs to treat recalcitrant biofilm infections.

#### **Key words:**

Drug conjugates, antineoplastic, antibiotic

## Biofilm formation on transcatheter aortic valve implants (TAVI) – an in vitro model

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### Introduction

Transcatheter aortic valve implantation (TAVI) is a percutaneous catheter-based method treatment of aortic stenosis as an alternative to open heart valve surgery. In cases of TAVI endocarditis, the treatment possibilities may be limited as surgical removal of the infected valve may be associated with a high risk in some of the elderly frail patients. The propensity of bacteria to form a biofilm on foreign material is assumed to be an important part of the disease process in TAVI endocarditis, but no studies on biofilm formation on TAVI valves have been performed. We hypothesized that *Staphylococcus aureus* and *Enterococcus faecalis* form biofilms on TAVI valves resulting in antibiotic tolerance and reduced cure rates.

### Methods

Pieces (100 mg) of TAVI valves were exposed to either species in vitro in LB-Krebs Ringer medium at 37 °C, with the bacterial count being assessed by culturing of sonicated TAVI pieces and broth at 0, 4, 18 and 24, and 48 hours after bacterial exposure. Scanning electron microscopy (SEM) was performed. Effects of ampicillin, gentamicin, and moxifloxacin, at 5 times minimal inhibitory concentration were tested. Antibiotics were added to biofilm aged 0 or 24 hours and the effects assessed.

### Results

Five minutes of exposure to either species at 100 colony-forming units (CFU)/ml established stable colonization in one out of six valve pieces. Exposure for 15 minutes established biofilm in all of valve pieces. SEM findings were consistent with biofilm formation and were suggestive of lower amounts of bacteria on the metal parts of the TAVI valves. The number of bacteria attached to the TAVI valves increased until 24 hours of incubation from less than 101 to a level of approximately 109 CFU/gram. The bacteria became more tolerant to antibiotics on the TAVI valves over time, with the bactericidal effect against 24-hour old biofilm being 101-108 times less effective than against 0-hour old biofilm depending on antibiotic.

### Conclusion

The results indicate that bacteria can adhere to TAVI valves with minutes after an exposure that is comparable to transient bacteremia in vivo, and that the bacteria rapidly gain biofilm properties, associated with significantly reduced antibiotic effect.

### Keywords:

Endocarditis TAVI *Staphylococcus* *Enterococcus*

## Self-assembled nanocoatings for combating implant related biofilm infections

Dr Seref Akay<sup>1</sup>, Manija Nazim<sup>1</sup>, Roudabeh Foroughian<sup>1</sup>, Prof. Oana Ciofu<sup>2</sup>, Assoc. Prof. Anan Yaghmur<sup>1</sup>

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<sup>2</sup>Costerton Biofilm Center, Department of Immunology and Microbiology, University of Copenhagen, Copenhagen, Denmark

### Background

Implant-associated infections (IAIs) represent a major health burden due to the complex 3D structural features of biofilms and their inherent tolerance to antimicrobial agents. To prevent the development of biofilms on implants, the design of efficient coatings with antifouling and antibacterial properties represents an attractive option. However, rapid degradation of coatings, non-optimal drug release, and toxicity are the main concerns that limit the usage of most investigated polymer coatings. In this study, we aim to develop self-assembled nanocoatings containing antibacterial agents for inhibiting bacterial attachment and biofilm formation on orthopedic implants. These nanocoatings are produced on the implants through the generation of thin dry lipid films that are converted to lyotropic non-lamellar liquid crystalline (LLC) nanostructures on exposure to excess water. These phases with unique nanostructural versatility, biocompatibility, and bioadhesive properties are attractive in the design of nanocoatings for the local delivery of antimicrobial agents.

### Methods

40  $\mu$ l of an ethanol solution containing colistin (COL), and glyceryl monooleate (GMO) was used to prepare thin and dry lipid films on 15-mm diameter stainless steel discs through the use of a spin coater. On exposure to excess water, these lipid films were hydrated in excess buffer to produce LLC nanocoatings. In addition to characterization studies (including SAXS) on LLC phases, the antibacterial activities of the self-assembled coatings were evaluated against the reference *P. aeruginosa* PAO1 strain.

### Results and Conclusions

The effects of lipid composition and antibiotic concentration on the structures of the LLC phases and antibacterial activity were investigated. It was observed a release of COL from LLC coated discs, that inhibited bacterial growth significantly compared to uncoated discs (OD600 values reduced from 0.814 (uncoated) to 0.095 (LLC coated),  $p < 0.05$ ). Similarly, the LLC coatings with a COL concentration of 0.145  $\mu$ g/cm<sup>2</sup> have been shown to decrease by log 2 the attachment of *P. aeruginosa* to the surface, compared to the uncoated discs. The designed self-assembled multifunctional LLC nanocoatings may provide potential use in implant functionalization for effectively combating biofilm development and associated infections.

### Acknowledgments

The study is funded by the European Union (Grant No: 101107704).

### Keywords:

Biofilm, coating, implant, liquid crystalline

# INNATE AND ADAPTIVE IMMUNE RESPONSES TOWARDS BIOFILM INFECTIONS

Invited speaker:

## Cystic Fibrosis lung infections: Immunological Features

Renan Marrichi Mauch

Characterised by defective or absent CFTR protein in the epithelial cells, and mucus accumulation in the lungs leading to persistent and incurable infections, mainly with *Pseudomonas aeruginosa*, the hereditary, multifactorial disease cystic fibrosis (CF) is one of the main models for the study of biofilms and the immune response against them. Neutrophils are the main players in this scenario, showing high degranulation, increased production of reactive oxygen species (ROS) and proteases, such as neutrophil elastase, sustained viability, and frustrated phagocytosis, culminating in DNA release through production of neutrophil extracellular traps (NETs) in a final attempt to kill the pathogen, and, ultimately, neutrophil necrosis. Altogether these events lead to the release of several toxic components in the tissue, high protease/anti-protease imbalance, along with increased mucus thickness, thus favouring *P. aeruginosa* persistence, perpetuated inflammation, and tissue damage.

In addition to the aberrant neutrophilic response, macrophages in CF are also hyperinflammatory but suboptimal, showing impaired pathogen recognition and phagocytosis, autophagy, signal transduction and termination, and decreased efferocytosis, meaning that their inflammation resolution capacity is also compromised.

In the adaptive immune compartment, there is a large body of evidence pointing to an increased IgG response against *P. aeruginosa* in CF as detected by serum-based methods. However, these IgG levels have shown to correlate with worse clinical status and increased lung damage, suggesting that they are defective, in line with studies showing defective IgG class switching, poor opsonisation of *P. aeruginosa*, and low avidity against alginate, the main component of the *P. aeruginosa* biofilm.

The literature is less consistent regarding the T cell response in CF, but usually suggests that the response to *P. aeruginosa* is characterised by high activity of CD4 T-helper-2 (Th2) and Th17 cells, and low numbers and activity of Th1 and regulatory (Treg) cells, which, altogether, may contribute to the dysregulated immune response seen in CF.

Other potential players in CF lung infections are mast cells, innate lymphoid cells, mucosal-associated invariant T (MAIT) cells, and  $\gamma\delta$  T cells; however, their roles require much more investigation.

Overall, the immune response in CF is not completely understood, and gaps to be covered include the spatial distribution of cells in the lung tissue, deeper immune phenotyping of CF blood and airway samples, investigation of the potential drivers of early inflammation in the absence of bacterial infection, and the impact of CFTR modulators in the immune response.

## Characterizing inter-species interactions upon in vivo-like conditions: *P. aeruginosa* boosts *S. aureus* adhesion and persistence on epithelial cells

Dr Maria Olivia Pereira<sup>1</sup>, Dr. Nuno Cerca<sup>1</sup>, Andreia Magalhães

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*Pseudomonas aeruginosa* and *Staphylococcus aureus* frequently colonize the same niche influencing Cystic Fibrosis (CF) pathogenesis. While interactions between *P. aeruginosa* and *S. aureus* have been extensively described using in vitro biofilm models, little is known about how bacteria interact with each other on infection models better resembling traits of CF environment. To evaluate whether key factors driving CF infections, such as bacteria-bacteria-host interactions, can shape *P. aeruginosa*-*S. aureus* dual species biofilms and have a role in inter-species interactions, in this work, an in vivo-like model was developed to promote and follow bacterial infection on lung epithelial cells, evaluate the adhesion, internalization, and intracellular survival of single- and dual-species consortia of *P. aeruginosa* and *S. aureus*. After 24 h of infection on A549 epithelial cells, an ecological shift was observed, from a *Pseudomonas*- (observed in the dual-species biofilms formed in an in vitro model) to a *Staphylococcus*-dominated community (in then in vivo-like model). A significant higher adhesion and successful invasion of *S. aureus* to epithelial cells while growing together with *P. aeruginosa* were also detected. The intracellular survival of *P. aeruginosa*-*S. aureus* consortia was also significantly higher than the observed in the mono-infections. These outcomes provide evidence that both invasion and intracellular replication occur during *P. aeruginosa*-*S. aureus* co-infection, which certainly contribute to the ability of both species in persisting during CF co-infection. Interestingly, the cytotoxic effects on host cells observed during mono-infection seem to be attenuated during co-infection, particularly for *P. aeruginosa* PA14 strain. Overall, these results suggest that the inter-species interactions occurring between *P. aeruginosa* and *S. aureus* while growing on epithelial cells are affected by their interplay with the host cells. Beyond highlighting novel insights that may help to comprehend the progression of CF-associated infections, this study demonstrated that microbial interactions are strongly affected by the host environment.

### Acknowledgements

Portuguese Foundation for Science and Technology(FCT) through the strategic funding of UIDB/04469/2020 unit and by LABELS, LA/P/0029/2020; FCT for AM grant (SFRH/BD/132165/2017).

### Keywords:

*P.aeruginosa*+*S.aureus* biofilms; in-vivo-like model; bacteria-host-interactions

## Extracellular host DNA contributes to pathogenic biofilm formation during periodontitis

Dr Olena Moshynets<sup>1</sup>, Dr Mariana Slobodanyk-Kolomoiets<sup>2</sup>, Dr Svitlana Khlebas<sup>2</sup>, Dr Iryna Mazur<sup>2</sup>, Ms Kateryna Rudnieva<sup>3</sup>, Dr Viktoria Potochilova<sup>4</sup>, Dr Olga Iungin<sup>5</sup>, Dr Geert Potters<sup>6</sup>, Dr Andrew Spiers<sup>7</sup>  
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### Background

Periodontal diseases are known to be associated with polymicrobial biofilms and inflammasome activation. A deeper understanding of the subgingival cytological (micro)landscape, the role of extracellular DNA (eDNA) during periodontitis, and the contribution of the host immune eDNA to inflammasome persistence, may improve our understanding of the mechanisms underlying severe forms of periodontitis. The aim of the study was to examine the realistic cytological landscapes of periodontitis and detailed analysis of eDNA found in such material.

### Methods

A polyethylene terephthalate (PET) film sampling protocol was developed for direct sampling procedure. Biofilm formed with standard strains *Escherichia coli* K12, *Klebsiella pneumoniae* ATCC 10031, *Pseudomonas aeruginosa* PA01, *P. fluorescens* SBW25, *Staphylococcus aureus* ATCC 29423 as well as the Ukrainian hospital isolates *K. pneumoniae* UHI 489 and UHI 117, *Streptococcus gordonii* UHI1, *S. sanguinis* UHI1 and *S. mitis* UHI1 were used as reference biofilms. Biofilm and cytological analysis were analyzed using a Leica TCS SPE Confocal system (CLSM). Fluorescence in situ hybridization (FISH) was used to visualize eDNA derived from eubacteria, streptococci and the *Bacteroides–Porphyromonas–Prevotella* (BPP) group in periodontal samples.

### Results

Subgingival biofilms developing on biologically neutral PET films placed in gingival cavities of patients with chronic periodontitis were investigated by CLSM. This allowed examination of realistic cytological landscapes and quantification of extracellular polymeric substances including carbohydrates, proteins, amyloids and eDNA, as well as comparison with several single-strain in vitro model biofilms produced by oral pathogens. Fluorescent stains were used to differentially visualise intracellular DNA and eDNA, amyloids and carbohydrates, and FISH analysis to identify eDNA derived from eubacteria, streptococci and the BPP group associated with periodontitis. Analysis of subgingival biofilm EPS revealed low levels of functional amyloids and high levels of eDNA which appears to be the main matrix component. However, bacterial eDNA contributed less than a third of the total eDNA observed, suggesting that host-derived eDNA released in neutrophil extracellular traps may be of more importance in the development of biofilms causing periodontitis.

### Conclusions

eDNA derived from host immunocompetent cells and activated at the onset of periodontitis may be a major driver of bacterial persistence and pathogenesis.

### Keywords:

Periodontitis, subgingival biofilms, eDNA

## Invited speaker:

### Does Biofilm specific immune responses exist? How specific are they?

Peter Østrup Jensen

*Costerton Biofilm Center, Department of Immunology and Microbiology, Faculty of Health Sciences, University of Copenhagen*

The immune response to infections has long been appreciated for preventing and clearing infections, as well as for supporting diagnostics. However, the immune response to infections is also associated with unfavorable effects resulting from activation of virulence, induction of tolerance to antibiotics and collateral tissue damage. Therefore, the activation of the immune response by the biofilm component in bacterial infections is of great importance.

The immune response is traditionally divided into the innate and specific response.

Neutrophils isolated from the blood are fully functional effector cells of the innate response and have essential bactericidal capacities, but they also may impose tissue destruction. The ability of neutrophils to discriminate biofilm have been examined according to the respiratory burst of isolated neutrophils in response to biofilm or planktonic bacteria. Both biofilm and planktonic bacteria activated the respiratory burst confirming the limited specificity of innate responses.

However, at low bacterial densities the respiratory burst was stronger against biofilm while the respiratory burst was weaker against biofilm at high bacterial densities. In addition, the polysaccharides of biofilm may determine the respiratory burst by the neutrophils indicating immunostimulatory properties of the biofilm matrix.

The reaction of the specific immune response is strongly dependent on the immunogenicity of the antigens that activates the generation of the clonal effector cells. The biofilm-induced activation of the T helper cells (Th) from the cellular arm of the specific immune response has been firmly demonstrated by the increased cytokine production by stimulated Th cells activated by biofilm infection in mice. The importance of the Th response is emphasized by the protective properties associated with the Th type 1 response against *Pseudomonas aeruginosa* biofilm lung infection in human as well as in mice.

Activation of B cells in the humoral arm of the specific immune response by biofilm is firmly demonstrated by the higher amounts of specific antibodies against alginate in blood from patients with mucoid *P. aeruginosa* biofilm lung infection compared to non-mucoid infection. Specific antibodies against *P. aeruginosa* antigens have been valuable for specific and sensitive detection of chronic biofilm lung infection. The induction of highly specific antibodies against biofilm-upregulated proteins in experimental *Staphylococcus aureus* osteomyelitis further underlines the ability of biofilm to induce specific immune responses.

In conclusion, biofilms have immunostimulatory properties with limited specificity and immunogenic properties with high specificity. Accordingly, the contributions of biofilm-stimulated immune responses during infections should be considered.

## Outsmarting the bladder: How the biofilm matrix of *P. aeruginosa* is more than just a protective mechanism in the urinary tract environment

Dr Arthika Manoharan<sup>1,2,3</sup>, A/Prof Greg Whiteley<sup>1,2,3,4,5</sup>, A/Prof Jim Manos<sup>1,2,3</sup>

<sup>1</sup>University Of Sydney, Camperdown, Australia, <sup>2</sup>Sydney Institute of Infectious Diseases, Camperdown, Australia, <sup>3</sup>Charles Perkins Centre, Camperdown, Australia, <sup>4</sup>Whiteley Corporation, Tomago, Australia, <sup>5</sup>Western Sydney University, Sydney, Australia

### Background

*P. aeruginosa* accounts for 20% of all nosocomial UTIs, often causing biofilm encrustation of indwelling catheters. Previous studies indicate that urine downregulates *P. aeruginosa* quorum sensing (QS), potentially increasing susceptibility to clearance by host immune system. Despite a lack of QS in urine, *P. aeruginosa* has adapted to thrive and establish biofilms in the urinary tract environment. In this study, we investigated the effect of urine on the *P. aeruginosa* biofilm matrix composition during UTIs and its immunogenicity to uroepithelial cells.

### Methods

Clinical uropathogenic *P. aeruginosa* biofilms were grown in the presence of synthetic urine in 1 cm catheter cross sections for 48 hrs at 37°C. The biofilm matrix was scraped and harvested using a series of gradient and chemical centrifugation steps. Protein, eDNA, polysaccharides and pyocyanin were quantified using standard methods. Mass spectrometry on the protein fraction defined urine mediated changes to the matrix proteome. To study the effect of the biofilm matrix on urothelial cellular inflammation, uroepithelial cell monolayers (ATCC 5637) were treated with different fractions of the harvested biofilm matrix without bacteria for 6, 12 and 24hrs. Pro-inflammatory cytokines and rates of wound healing were quantified.

### Results

Urine grown biofilm matrices, showed a >3-fold increase in protein concentrations, and a significant reduction in polysaccharides ( $p < 0.01$ ), compared to nutrient broth controls. The protein fraction elicited the strongest inflammatory response from urothelial cells, with a significant increase in IL-6, IL-8 and IL-1b in response to infection (>2-4 fold compared to untreated controls,  $p < 0.05$ ) and a significant reduction in rate of wound healing in vitro. No significant differences were recorded between the polysaccharide and eDNA fractions.

### Conclusion

Urine significantly influences the composition and immunogenicity of the *P. aeruginosa* biofilm matrix. The biofilm matrix has a larger protein component than extracellular polysaccharides compared to biofilms grown in nutrient broth. The matrix is also highly immunogenic to uroepithelial cells, indicating that its role in UTIs extends beyond bacterial protective mechanisms. Studies are currently underway to characterize the matrix proteome and biofilm transcriptome. This work highlights the importance of optimizing treatment options to target the biofilm matrix and reduce recurrent catheter infections.

### Keywords:

UTIs, *P. aeruginosa*, matrix immunogenicity, urine

## Delayed neutrophil recruitment to the tissue-implant-interface is seen within the first hours following insertion of a contaminated abiotic implant in mice

Dr. Marie H. Hansen<sup>1</sup>, Dr. Philip S. Stewart<sup>2</sup>, Dr. Timothy Borgogna<sup>2</sup>, Professor Thomas Bjarnsholt<sup>3</sup>, Professor Louise K. Jensen<sup>1</sup>

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### Background

Medical implants are inserted under strict sterile conditions, yet bacterial contamination is a risk and can lead to chronic biofilm implant-related infections. Why doesn't the immune system eradicate the contamination? Maybe immune cells, particularly neutrophils, reach the implant surface too late allowing time for surface biofilm formation. This study aimed to investigate whether there is a delayed recruitment of neutrophils to the tissue-implant-interface in a murine model.

### Material and Methods

Female mice were anesthetized, allowing subcutaneous placement of a titanium disc (5 µm in diameter and 1 µm thick) on their back. The disc was inoculated with either a high bacterial dose (1x10<sup>8</sup> CFU), low bacterial dose (2.5x10<sup>5</sup> CFU), or sterile 0.9% saline, respectively. *Staphylococcus aureus* (USA300 AH1726) was used as inoculum. Four mice from each group were euthanized at 4-, 24-, and 72-hours post-surgery. Local tissues were biopsied for histology. Histologically, neutrophil infiltration in subcutis and at the implant-tissue-interface was scored as absent (0), minor (1), moderate (2), or massive (3). Furthermore, artificial intelligence (AI) pathology was used to identify the tissue-implant interface (34.4 µm) and automatically count the number of immune cells inside.

### Results

At 4 hours, infected mice (combined low and high dose) showed a significant (P=0.0001) increase in subcutis neutrophil score (median=2) in comparison to non-infected mice (median=0). In contrast, both groups had a very low, non-significant neutrophil infiltration score at the tissue-implant-interface (infected: median=0.5, non-infected: median=0). After 24- and 72-hours the subcutis and interface neutrophil scores were identical for all groups. For all time points the high dose inoculum showed a tendency towards more neutrophil infiltration in comparison to the low dose. AI pathology of the implant-tissue-interface confirmed the semiquantitative findings.

### Conclusion

Recently it has been shown that an in vivo surface biofilm is formed within 3 hours. The present preliminary study showed that neutrophil recruitment to the implant surface was delayed in the first hours after implantation in comparison to subcutis and this phenomenon might be an overlooked component in development of implant-related infections.

### Keywords:

Neutrophils, implant-associated infection, histology, murine.

Friday 28 June

## MIXED SPECIES BIOFILMS – IN/ON HOST AND OUTSIDE

Invited speaker:

### Imaging of dental biofilms

Sebastian Schlafer

Dental biofilms are the causative agents of caries, gingivitis and periodontitis and a prime example of a multispecies microbial community. In contrast to many other medically relevant biofilms, structurally intact dental biofilms can be collected noninvasively, and they may therefore serve as a model to study a variety of fundamental processes in microbial biofilms.

There is a long history of structural imaging of dental biofilms using, e.g., electron microscopy or fluorescence in situ hybridization combined with confocal laser scanning microscopy. Most studies have focused on the microbial inhabitants of dental biofilms and contributed to our understanding of architectural features, colonization patterns and potential interactions between putative pathogens. The past decade, however, has seen a growing interest in the biofilm matrix, and a series of elegant studies has highlighted its importance for biofilm virulence. Along with the focus shift towards the biofilm matrix, several methods for chemical imaging of important solutes have been developed or adapted to dental biofilms. Ratiometric approaches or fluorescence lifetime imaging allow for the three-dimensional mapping of, e.g., pH or oxygen levels in biofilms. When applied in concert with structural imaging, these techniques have the potential to greatly enhance our understanding of biofilm metabolism and virulence at the microscale.

## Invited speaker:

### Impact/mechanisms of Interspecies biofilm interactions for infection severity and treatment

Chelsie Ambruster

Urinary tract infections (UTIs) are among the most common infections worldwide, of which up to 80% are catheter associated (CAUTI). Catheter insertion facilitates bacterial colonization through a variety of means, resulting in a 3-8% incidence of bacteriuria and persistent polymicrobial colonization during long-term catheterization. Through longitudinal assessment of individuals with long-term catheters, we identified two common CAUTI pathogens, *Proteus mirabilis* and *Enterococcus faecalis*, as persistent co-colonizers. We show that these two species co-localize within catheter biofilms and in bladder tissue during experimental infection, and that co-colonization enhances biofilm biomass, antibiotic resistance, and infection severity. Through compositional and proteomic biofilm analyses, we determined that the increase in biofilm biomass stems from an increase in the protein fraction of the polymicrobial biofilm, with specific enrichment in proteins associated with ornithine and arginine metabolism in polymicrobial biofilms compared to single-species biofilms. We show that L-ornithine secretion by *E. faecalis* promotes arginine biosynthesis in *P. mirabilis*, and that disruption of this metabolic interplay abrogates the biofilm enhancement we see *in vitro* and leads to significant decreases in infection severity and dissemination in a murine CAUTI model. Thus, modulating metabolic interplay between these species could potentially disrupt persistent colonization and progression to severe disease.

## STRATEGIES FOR FIGHTING BACK BIOFILMS

Invited speaker:

### Hyperbaric oxygen therapy (HBOT) and endocarditis - clinical experience

Christian Lerche

Infective endocarditis (IE) is a life-threatening infection of the heart valves, with an in-hospital mortality rate of 20% and a 1-year mortality rate of up to 40% for left-sided IE. Despite medical advancements, IE remains associated with a high risk of mortality, with no significant improvement in survival rates over the past three decades. New treatment strategies are therefore urgently needed to improve the morbidity and mortality of IE patients. IE is characterized as a biofilm infection on either the native valve endothelium or prosthetic material within the heart, necessitating prolonged high-dose antibiotic treatment for at least 4 to 6 weeks. In this presentation, the known and potential beneficial aspects of hyperbaric oxygen therapy (HBOT) will be elaborated upon in the context of biofilm infections and IE. The current evidence from in vitro studies, preclinical experimental endocarditis studies, and preliminary results from a clinical feasibility study of hyperbaric oxygen treatment for left-sided IE will be presented.

## The importance of superoxide anion for Escherichia coli biofilm removal using plasma-activated water

Mrs Binbin Xia<sup>1</sup>, Dr Heema Kumari Nilesh Vyas<sup>1,2</sup>, Dr Renwu Zhou<sup>1,3</sup>, Dr Tianqi Zhang<sup>1</sup>, Dr Jungmi Hong<sup>1</sup>, Dr Joanna G. Rothwell<sup>4</sup>, Prof Scott A. Rice<sup>5</sup>, Prof Dee Carter<sup>2,4</sup>, Prof Kostya (Ken) Ostrikov<sup>6</sup>, Prof Patrick J. Cullen<sup>1</sup>, Dr Anne Mai-Prochnow<sup>1</sup>

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Microbial biofilms cause contaminations in different environmental settings, including pipelines, filters, membranes, food and processing infrastructure. They ultimately pose a major risk to human health and necessitate costly cleaning and repair. Cold plasma, a partially ionised gas, and plasma-activated water (PAW) exhibit powerful disinfectant activity for fighting back biofilms. However, the optimal generating conditions, such as the choice of gas used to produce PAW, remain unclear. Here, a range of different PAWs were generated from argon, nitrogen, air, and oxygen in a plasma bubble spark discharge (BSD) reactor capable of directly treating Escherichia coli (ATCC 25922) biofilms in situ. We measured the reactive oxygen and nitrogen species (RONS) (H<sub>2</sub>O<sub>2</sub>, NO<sub>3</sub><sup>-</sup>, NO<sub>2</sub><sup>-</sup>) in PAW and the excited species via optical emission spectroscopy (OES). PAW generated using oxygen (PAW-O<sub>2</sub>) was the most effective and completely removed E. coli biofilms on stainless steel surfaces. Confocal microscopy demonstrated that PAW treatment removed most biofilm cells from the surface with only a few dead cells remaining. We demonstrated that intracellular ROS level increases significantly in the PAW-O<sub>2</sub>-treated biofilms. Using molecular scavengers, we showed that superoxide anion radical ( $\bullet$ O<sub>2</sub><sup>-</sup>) played a critical role in the inactivation of E. coli biofilms. We also confirmed the generation of  $\bullet$ O<sub>2</sub><sup>-</sup> in the PAW-O<sub>2</sub> via electron paramagnetic resonance (EPR) spectrometry. The potential chemical reactions that occurred in PAW were hypothesized via optical emission spectra (OES). Our results demonstrate the importance of input gas and plasma operating conditions to maximise effective RONS production for optimal biofilm removal under real environmental and industry-relevant conditions.

### Keywords:

Plasma-activated water; Biofilm removal; Escherichia-coli

## Repetitive combined doses of bacteriophages and gentamicin protect against *Staphylococcus aureus* implant-related infections in *Galleria mellonella*

Dr Gopala Krishna Mannala<sup>1</sup>, Pr Markus Rupp<sup>1</sup>, Dr Nike Walter<sup>1</sup>, Dr Raphaelle Youf<sup>1</sup>, Dr Susanne Bärthl<sup>1</sup>, Pr Martijn Riool<sup>1</sup>, Pr Volker Alt<sup>1</sup>

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### Introduction

Bacteriophages are viruses that infect and replicate inside bacteria and finally release from the host through lysis. Recently, we demonstrated the efficacy of the *Staphylococcus aureus*-specific phage 191219 in vitro. However, in the *Galleria mellonella* biofilm implant-infection model, a single dose of phages did not improve the survival rate of the larvae

### Objectives

Here, we aim to evaluate the effects of repetitive doses of the combination of the *Staphylococcus aureus*-specific phage 191219 and gentamicin in the prevention and treatment of hematogenous and early-stage biofilm implant-related infections in *Galleria mellonella*, respectively.

### Methods

To mimic the hematogenous infection, *G. mellonella* larvae were implanted with a Kirschner (K)-wire and infected with *S. aureus* EDCC 5055 after 24h. Subsequently, phages, gentamicin, or a combination of both, were administered. For the early-stage biofilm implant infection model, the K-wires were pre-incubated in a *S. aureus* suspension for 30 min before implantation. After 24h, the larvae received a single dose of phages, gentamicin or their combination. In both models, the larvae also received daily doses of phages and/or gentamicin up to 5 days. The survival rate of larvae was monitored and, after 2 days of repetitive administration of phages and/or gentamicin, quantitative cultures of the bacteria in the tissue of larvae as well as on the implant surface were assessed.

### Results and Conclusions

In the hematogenous infection model, a single combined dose of phages and gentamicin, and repetitive injections with gentamicin alone or in combination with phages resulted in significantly improved survival rates. In the early-stage biofilm infection model, only repetitive combined administration of phages and gentamicin led to a significantly increased survival. Additionally, a significant reduction in number of bacteria was observed in the larvae after receiving repetitive doses of phages, gentamicin or their combination in both infection models. Based on our in vivo results, a single dose of the combination of phages and gentamicin is sufficient to prevent a hematogenous *S. aureus* implant-related infection, whereas gentamicin needs to be administered daily for the same effect. To treat early-stage *S. aureus* implant-related infection, repetitive doses of the combination of phages and gentamicin are required.

### Keywords:

in vivo, gentamicin, phages, implant

## Invited speaker:

### Disperazol - new Drug on the Block – Preclinical Aspects

Michael Givskov 1 \*, Michael Graz 1 $\alpha$ , Tim Tolker-Nielsen 1, Louise Dahl Hultqvist 1, Jens Bo Andersen 1, Charlotte Uldahl Jansen 2, Carl Martin Nilsson 3, Morten Rybtke 1, Tim Holm Jakobsen 1, Thomas Eiland Nielsen 1, Klaus Qvortrup 4, Claus Moser 1,5, Katrine Qvortrup 2, and Urs Jenal 6 and his team.

*\*Presenter  $\alpha$  Business Developer*

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Disperazol has been developed to become a therapeutic candidate that is co-administered with standard of care (SoC) antibiotics for efficient treatment of recalcitrant *Pseudomonas aeruginosa* infections. With Disperazol, we have defined a new class of non-lethal antimicrobial, operating through the universal c-di-GMP messenger system of Gram-Negative bacteria. Our primary indication is hospital-acquired, catheter associated urinary tract infections (CAUTI). About 75% of UTIs acquired in hospitals are due to biofilms formed on catheters which are installed in up to 25% of hospitalised patients. 23% of these biofilms are positive for *P.aeruginosa*, indicating that *P.aeruginosa* CAUTIs have become a significant healthcare burden.

Within the bacteria, high levels of c-di-GMP sustain the biofilm life-mode, whereas a sudden drop in c-di-GMP activates several enzymatic processes causing biofilms to dismantle and liberate antibiotic susceptible bacteria. This is what Disperazol does to *P.aeruginosa*. In contrast to their planktonic counterparts, biofilm bacteria show a remarkable, overarching capacity to withstand multiple classes of antibiotics. The insight gained is that the biofilm life mode is paralleled by an armoury of molecular resistance-tolerance mechanisms.

Our focused approach to deal with this is to “drug” the c-di-GMP signalling pathway and turn bacteria into the planktonic, low AMR mode. A recent breakthrough with Urs Jenal’s laboratory in Basel established the molecular basis of Disperazol’s biological activity; the drug binds directly to and specifically activates the phosphodiesterase activity of BifA, one of 41 enzymes responsible for modulating the intracellular c-di-GMP level.

When Disperazol induced biofilm dismantling is coupled with SOC treatments, it mitigates the overall AMR properties of the infection. This has been convincingly demonstrated in vivo, using a murine CAUTI model with *P.aeruginosa*-coated catheters. In this model system, a combination of 3 $\mu$ g Disperazol/g BW and 1 $\mu$ g Ciprofloxacin/g BW administered orally to mice, proved to be 100-fold more efficient than oral Ciprofloxacin administration alone.

## 5-fluorocytosine (5-FC) inhibits biofilm formation and virulence in uropathogenic *Escherichia coli* strains and potentiates $\beta$ -lactams antimicrobial activity against pre-formed biofilms

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### Background and aims

Biofilm growth increases bacterial tolerance to antibiotics, thus posing a challenge for treatment of biofilm-associated infections in clinical settings. The FDA-approved antimycotic 5-fluorocytosine (5-FC) possesses antibiofilm activity against laboratory *Escherichia coli* strain MG1655, reducing curli fibre production with a negligible effect on bacterial growth. In this project, we aimed at determining whether 5-FC antibiofilm activity is preserved in clinically relevant *E. coli* strains and test possible synergies with antibiotics in treating pre-formed *E. coli* biofilm.

### Methods

The ability of subinhibitory concentrations of 5-FC to inhibit biofilm formation, disrupt pre-formed biofilm, and modulate virulence determinants was tested on clinical isolates (Rigshospitalet, Denmark) of uropathogenic *E. coli* (UPEC) strains using the standard crystal violet based biofilm adhesion assay and minimum biofilm eradication concentration (MBEC) assay, and by measuring through RT-qPCR the expression of curli fibres (*csgB*, *csgA*), type-1 fimbriae (*fimA*) and P fimbriae (*papC*) and  $\alpha$ -hemolysin (*hlyA*) encoding genes. The effect on viability of cells within pre-formed biofilm was monitored using fluorescein diacetate assay on biofilm treated with or without 5-FC and specific antibiotics.

### Results

5-FC was able to inhibit biofilm formation in all UPEC strains, albeit to a different extent. Gene expression analysis suggested that in addition to inhibiting curli gene expression (*csgB*, *csgA*), 5-FC significantly represses transcription of the virulence genes *fimA*, *papC*, *hlyA*. Though 5-FC could not disrupt pre-formed UPEC biofilm, the combination of 5-FC with ceftazidime, aztreonam, and piperacillin resulted in a striking reduction of viability of bacteria dwelling inside pre-formed biofilm. This synergistic effect is specific for  $\beta$ -lactams, as the same results were not obtained with the combination of 5-FC with fluoroquinolones and aminoglycosides.

### Conclusions

5-FC shows anti-biofilm and anti-virulence activity in uropathogenic *E. coli* strains by reducing expression of curli fibres, type-1 and P fimbriae, and  $\alpha$ -hemolysin. When used in combination with  $\beta$ -lactams, 5-FC can kill bacteria within pre-formed UPEC biofilm known to be extremely resilient to antibiotics treatment. Overall, our data suggests that 5-FC is an excellent candidate for preventing and treating bacterial infections associated with the presence of recalcitrant biofilms.

### Keywords:

UPEC, 5-fluorocytosine, Synergistic antimicrobial activity

## Dual function of N-acetyl cysteine in eradicating *P. aeruginosa* catheter-associated UTIs

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### Background

*P. aeruginosa* is a key uropathogen accounting for 20% of all catheter-associated urinary tract infections. They are known to form antibiotic resistant biofilms on indwelling catheters and within the bladder epithelium. Though associated with extensive catheter encrustation, there is a lack of novel treatment strategies to address *P. aeruginosa* UTIs. Thus, we investigated the potential of N-acetyl cysteine, a thiol antioxidant with established antibiofilm properties.

### Methods

Silicon catheter encrustation was investigated in the presence of NAC using an in vitro glass bladder model, where a 2-way Foley catheter was “infected” with *P. aeruginosa*. Artificial urine with NAC was pumped through the catheter and “infection” was allowed to run for 120hrs. Inductively coupled plasma mass spectrometry analysis was used on catheter eye sections with differences in elemental profiles of biofilms in catheters compared. Furthermore, the influence of NAC treatment on the inflammatory response of urothelial cells to infection was quantified by infecting ATCC 5637 urothelial cells with *P. aeruginosa* in the presence of NAC and collecting the supernatant for ELISA analysis. ROS production and wound healing was also quantified in these samples.

### Results

For the first time, we observed that *P. aeruginosa* forms thick mucoid non-crystalline biofilms that can block catheters. Additionally, NAC significantly prevented *P. aeruginosa* encrustation of catheters, reducing biofilm bacteria by  $> 4\log_{10}$  ( $p < 0.05$ ), and urothelial cell invasion ( $p < 0.01$ ), and inhibited substratum attachment. Moreover, the elemental profiles of NAC treated biofilms significantly varied, with stark decreases in  $\text{Ca}^{2+}$ , P, K, Na ions concentrations. Furthermore, we saw a significant reduction in IL-6, IL8 and TNF- $\alpha$  responses by urothelial cells to *P. aeruginosa* infection in presence of NAC, as well as an enhanced rate of wound healing in these cells ( $> 0.1$  mm/hour).

### Conclusion

NAC is a well characterised non-toxic antibiofilm agent with potent antibiofilm and anti-inflammatory capabilities in the bladder environment. With dual inhibitory capacities, NAC potentially allows for targeted CA-UTI eradication of “fugitive” *P. aeruginosa* biofilm bacteria anywhere in the infected catheterised bladder and could be a key adjuvant alongside antibiotic therapy to improve clinical outcomes.

### Keywords:

N-acetyl cysteine, UTIs. Inflammation

# SPATIAL BIOFILM ORGANIZATION

Invited speaker:

## Predicting interactions in multispecies biofilms

Ákos T. Kovács

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There is a strong interest in the field of microbial ecology to predict interactions between microorganisms, and determine whether microbial isolates will promote the growth of each other or compete for resources. Numerous studies have attempted to map interaction networks, however, those experiments are based on high-diversity synthetic communities, limiting taxonomy-based prediction of the interaction outcome. Here, we applied a high throughput screen using 720 *Pseudomonas* isolates and tested their impact on the biofilm formation of *Bacillus subtilis*. We aimed to determine whether the majority of *Pseudomonas* would promote or inhibit the biofilm of *B. subtilis* in pairwise co-cultures. We found that *Pseudomonas* taxonomy is a good predictor of interaction outcome and only a minority of *Pseudomonas* isolates promotes *Bacillus* biofilm establishment. Importantly, using these taxonomic predictions, we could validate the positive or negative interactions between selected type strains of *Pseudomonas* and *B. subtilis*. To increase the complexity in ecological predictions, we also tested the power of genome scale metabolic modeling to identify keystone members of synthetic biofilm communities. These approaches revealed beneficial and detrimental members of a synthetic community depending on their metabolic references, which could be validated using experimental tests, highlighting the predictability of ecology from genome scale metabolic modeling, from genomes to interactions.

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*Zhang R, Kovács ÁT (2022) Bacillus velezensis stimulates resident rhizosphere Pseudomonas stutzeri for plant health through metabolic interactions. ISME Journal 16:774–787*

## Complementary exopolysaccharide-targeting antibodies reveal unique biofilm features and synergize to improve antibacterial efficacy

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### Background incl. aims

A key component of many pathogenic biofilms is the exopolysaccharide polysaccharide intercellular adhesin (PIA), a partially deacetylated polymer of poly-N-acetyl glucosamine (PNAG). The nature, extent, and location of deacetylation in natively expressed PIA is not well understood in part due to a lack of suitable research tools. As part of a broader effort to address the lack of high-quality anti-glycan antibodies (mAbs), we used a glycan microarray to screen a panel of human mAbs. From this panel, we identified novel anti-glycan mAbs, including TG10 which was highly selective for highly deacetylated PNAG (dPNAG). We sought to structurally define the epitope and evaluate TG10 in vitro and in vivo.

### Methods

TG10 epitope specificity was assessed by evaluation on glycan microarrays, X-ray crystallography, and high resolution *S. epidermidis* biofilm confocal microscopy. In vitro and in vivo efficacy was evaluated in OPK assays and in a lethal *S. aureus* challenge in a murine model.

### Results

Using microarrays and X-ray crystallography, we demonstrate that TG10 targets deacetylated forms of PIA whereas F598, an antibody in phase II clinical trials, preferentially binds highly acetylated forms. Additionally, we demonstrate that Staphylococcal biofilms contain distinct regions of high and low N-acetylation, rather than polysaccharides with intermittent deacetylation throughout the biofilm. In addition to revealing important features of biofilm architecture, the discovery suggested that PNAG specific antibodies, such as F598, will not opsonize the entire cell population in the biofilm. We hypothesized that combining the dPNAG-specific antibody TG10 with the PNAG-specific antibody F598 would enhance efficacy. TG10 and F598 synergistically increased in vitro and in vivo activity, providing 90% survival in a lethal *S. aureus* challenge murine model.

### Conclusions

The results reveal unique features of PNAG-dependent biofilms and provide an improved clinical strategy for targeting exopolysaccharides.

### Keywords:

PIA, PNAG, anti-glycan mAbs, Confocal

## Early development of *Staphylococcus aureus* fibrin biofilms

Miss. Safae Oukrich<sup>1</sup>, Mariël Leon-Grooters<sup>1</sup>, Dr. Gert van Cappellen<sup>2</sup>, Dr. Johan Slotman<sup>2</sup>, Prof. Dr. Gijsje Koenderink<sup>3</sup>, Dr. Willem van Wamel<sup>4</sup>, Prof. Dr. Moniek de Maat<sup>5</sup>, Dr. Klazina Kooiman<sup>1</sup>, Dr. Kirby Lattwein<sup>1</sup>

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### Background

Infective endocarditis (IE) is a severe and life-threatening infection caused by microbial colonization of the endocardium, predominantly affecting the heart valves. The single most common infecting microbe causing IE is *Staphylococcus aureus* (*S. aureus*). Within the cardiovascular system, surface-bound and secreted coagulases produced by *S. aureus* lead to fibrin-based biofilms, which provide strong adherence to intravascular structures, defense against the immune system, and resistance to antimicrobial treatment. However, the fibrin formation process in *S. aureus* IE remains poorly understood. This study investigated the early development of *S. aureus* fibrin biofilms.

### Methods

Clinically isolated coagulase-positive strains *S. aureus* 25268 and 50825, and coagulase negative strains *S. aureus*  $\Delta$ coa and *Streptococcus gordonii* (*S. gordonii*), were grown in tryptic soy broth (TSB), Iscove's Modified Dulbecco's Medium (IMDM), and pooled human plasma, with or without porcine heart valves. Bacterial growth, metabolic activity (using CalScreener, SymCel), and bacterial fibrinogen utilization was measured over 24h at 37°C. Confocal microscopy imaging was used to visualize and track biofilm development. For visualization, fluorophores SYTO9 and propidium iodide were used for bacteria and fibrinogen conjugate Alexa Fluor 647 for fibrin.

### Results

Coagulase-positive *S. aureus* 25268 had more growth in TSB and human plasma than coagulase-negative strains, but similar growth in IMDM as *S. aureus*  $\Delta$ coa. The *S. gordonii* showed limited bacterial growth when assessed using absorbance bacterial growth assay, while metabolic activity was detected through microcalorimetry. Peak metabolic activity for all isolates was highest in TSB, lowest in human plasma. When porcine valves were present, strain-dependent alterations in time to reach peak metabolic activity were observed. Confocal imaging revealed fibrin-based biofilm development exclusively in the coagulase-positive *S. aureus* strain. Initial fibrin strands appeared after  $1.8 \pm 0.3$ h and the total fibrin volume subsequently increased  $69.0\% \pm 6.0\%$  between 2 and 6h. These kinetics aligned with the fibrinogen utilization data. Variations in fibrin network porosity and volume between biofilms developed by coagulase-positive *S. aureus* strains were observed.

### Conclusions

Fibrin-based biofilm formation is mediated by *S. aureus* coagulase and takes place within several hours after exposure to a human plasma environment. This study provides insights into early *S. aureus* IE pathogenesis.

### Keywords:

Endocarditis; *Staphylococcus aureus*; Fibrin(-ogen); Staphylocoagulase

Invited speaker:

## Spatial transcriptomics in biofilms

Jintao Liu, PhD

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Microbial communities often display region-specific properties, which give rise to complex interactions and emergent behaviors that are critical to the homeostasis and stress response of the communities. However, systems-level understanding of these properties still remains elusive. We established RAINBOW-seq and profiled the transcriptome of *Escherichia coli* biofilm communities with high spatial resolution and high gene coverage. We uncovered three modes of community-level coordination, including cross-regional resource allocation, local cycling, and feedback signaling, which were mediated by strengthened transmembrane transport and spatially specific activation of metabolism. As a consequence of such coordination, nutrient limited region of the community maintained unexpectedly high level of metabolism, enabling it to express many signaling genes and functionally unknown genes with potential sociality functions. Our work provides an extended understanding of the metabolic interplay in biofilms and presented a new approach of investigating complex interactions in bacterial communities on the systems level.

## A Novel Multiplex mRNA PNA-FISH for Studying Gene Expression and Spatial Organization in Biofilms

Miss. Ana Barbosa<sup>1,2</sup>, Mr Nuno F. Azevedo<sup>1,2</sup>, Mrs Darla M. Goeres<sup>1,2,3</sup>, Mrs Laura Cerqueira<sup>1,2</sup>

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Fluorescence in situ hybridization using Nucleic Acid Mimics (NAM-FISH) is a powerful and versatile molecular technique that uses fluorescently-labelled NAMs, such as peptide nucleic acids (PNA), to penetrate microbial cells and hybridize specific rRNA sequences. This technique provides information about the localization of individual cells in multispecies biofilms and their three-dimensional spatial distribution without disturbing the biofilm structure. By adapting PNA-FISH to target mRNA with high-resolution imaging, it is possible to study gene expression in single cells either in free-living or in biofilm states, combining spatial and morphogenetic information. Under the scope of the ERA Chair e.Biofilm Project, this work aims to explore the use of mRNA PNA-FISH to study the regulatory network involved in the biphasic life cycle of *Legionella pneumophila*, helping to clarify the biofilm functionality and *L. pneumophila* ecology. Five PNA probes targeting the genes involved in the biphasic life cycle of *L. pneumophila* were developed using bioinformatics tools that consider sequence size, % GC, Tm, and specificity. Probes were tested in *L. pneumophila* serogroup 1 WDCM planktonic cells grown in a buffered yeast extract (BYE) medium. A bacterial growth curve determined the best time point for the probe optimization and bacteria growth phase. The optimal hybridization signal was obtained at 60°C after 30 h of bacterial growth for the *mip* (virulent gene) and *proA* (housekeeping gene). Future work includes optimizing the additional mRNA probes. This study highlights the potential of a model that uses mRNA PNA-FISH to study the regulatory network of *L. pneumophila* biofilms. This model can provide essential data on gene expression in individual cells, both in the planktonic and sessile states, while also recording functionality and spatial information.

### **Keywords:**

PNA-FISH, *Legionella*, biofilms, spatial organization

## Spatiotemporal dynamics of ECM production in *Pseudomonas* sp. IsoF govern biofilm topography

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Bacterial biofilms are structured communities encased in a self-produced extracellular matrix (ECM) that promotes surface colonization and increases stress resistance. While the key components and roles of the ECM for the biofilm lifestyle are often known, their spatiotemporal production and dynamics remain difficult to study, owing to a lack of suitable in vivo visualization methods.

The biocontrol rhizobacterium *Pseudomonas* sp. IsoF (IsoF) forms solid biofilms in classic experimental setups, including flow cells. In this study we demonstrate that under native c-di-GMP levels the IsoF ECM consists exclusively of cellulose. By the use of a new fluorescent stain in combination with submicrometric 3D imaging we were able to track matrix dynamics in real-time. Using this approach, we were able to follow biofilm development from initial colonists to mature macro-communities. Our flow-cell experiments show that 1) upon surface adhesion immobilized single IsoF cells and their progeny rapidly encase themselves into a self-produced ECM; 2) the progeny at the periphery of microcolonies depart and populate vacant spaces following the same program; 3) extracellular polysaccharide fibers produced in adjacent microcolonies interact with each other, providing stability to the matrix until 4) there is a complete occupancy of the entire available surface. Our data show that the stability of the IsoF biofilm depends on the cellulose matrix and that c-di-GMP-controlled cellulose production and self-assembly drive biofilm structural development.

The high experimental reproducibility of IsoF biofilm development in discrete settings (flow-cells, agar colonies and pellicle biofilms) makes this bacterium a safe and reliable model for studying multicellular communities.

### **Keywords:**

biofilm, extracellular matrix dynamics, *Pseudomonas*

## RESISTANCE MECHANISMS OF BIOFILMS

Invited speaker:

### Evolution of antibiotic resistance during the treatment of biofilms

Christophe Beloin

In many environments, bacteria thrive by colonising surfaces and forming dense communities known as biofilms. Although bacterial biofilms play important ecological roles, their development on medical devices and on some human mucosal surfaces is a major source of infection in clinical settings. Indeed, biofilms exhibit a high but reversible level of tolerance to antibiotics, leading to frequent therapeutic failures. While the link between this tolerance and therapeutic failure is reasonably well accepted, the possibility that this biofilm tolerance could also be a stepping stone for the emergence of antibiotic resistance has been little considered until recently. Several studies published in the last 5 years, in particular using adaptive experimental evolution, support the hypothesis that biofilms have specific characteristics that can promote the emergence of antibiotic resistance under certain treatment regimens. I will present our efforts to explore the interplay between biofilm tolerance and true antibiotic resistance and to increase the relevance of our in vitro and in vivo models to support the need for increased awareness of the importance of biofilms in the clinical management of infections.

## Transfer and persistence of plasmids in biofilm

Henriette Lyng Røder<sup>1</sup>, Eleni Cristidi<sup>2</sup>, Cristina Amador<sup>1</sup>, Urvish Trivedi<sup>1</sup>, Jakob Russel<sup>1</sup>, Kasper N. Kragh<sup>1</sup>, Tim Tolker-Nielsen<sup>1</sup>, Thomas Bjarnsholt<sup>1</sup>, Samra Music<sup>1</sup>, Asmus K. Olesen<sup>1</sup>, Birte Svensson<sup>3</sup>, Jonas S. Madsen<sup>1</sup>, Jakob Herschend<sup>1</sup>, Jan-Ulrich Kreft<sup>2</sup>, Mette Burmølle<sup>2</sup>

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### Background

Bacteria can use horizontal gene transfer (HGT) to rapidly adapt to changes in their environment by directly transferring mobile elements like e.g. plasmids. This can ensure their survival even under otherwise detrimental conditions such as exposure to antimicrobial agents. Biofilms are the primary lifestyle of bacteria and they have been proposed to be a hotspot for HGT because of the high cell density and proximity, and structural stability. Here we set out to study the transfer and persistence of plasmids in biofilm communities.

### Methods

Two different dual-labelled systems were used for detection of plasmid transfer or stability, respectively. Flow cytometry and confocal laser scanning microscopy was used for detection. Proteomics and knock-out mutants were used to confirm observations of increased plasmid uptake as well as the use of individual-based modelling to confirm or eliminate possible underpinning mechanisms.

### Results

The results showed how biofilms can indeed act as spatiotemporal reserves for plasmids, allowing them to persist even under non-selective conditions. We further observed how the dynamic structure of biofilms directly impact the number of plasmids that are retained. We also identified a new factor impacting the transfer of plasmids – flagella, which are required for motility by many bacteria. We demonstrate that their absence or altered activity can lead to enhanced plasmid dissemination. Furthermore, we demonstrate the utility of mathematical modelling to test hypothetical explanations.

### Conclusion

Our findings identifies biofilms as conducive towards maintaining genetic diversity as well as shedding light on the complex effects of flagella on bacterial conjugation in biofilms.

### Keywords:

biofilm, plasmid, conjugation, flagella

## Unraveling adaptive strategies: Transcriptomic insights into *Pseudomonas aeruginosa* biofilm and planktonic populations evolving under sub-lethal levels of ciprofloxacin

Dr Marwa N. Ahmed<sup>1</sup>, Dr Janne G. Thöming<sup>2</sup>, Prof Dr Susanne Häussler<sup>2</sup>, Prof Dr Oana Ciofu<sup>3</sup>

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### Background

Chronic bacterial infections, exacerbated by antibiotic resistance, pose a significant health challenge, particularly in the context of *Pseudomonas aeruginosa* (Pa) infections in Cystic Fibrosis (CF). The formation of biofilms by Pa complicates treatment, shielding bacteria and contributing to antibiotic resistance. Ciprofloxacin is a commonly used antibiotic for Pa in CF patients. Experimental evolution studies of PAO1 wild-type (WT) and  $\Delta$ katA in sub-inhibitory ciprofloxacin conditions revealed that biofilm growth promotes the development of low-level resistant mutants. Oxidative stress was found to increase mutagenesis and resistance development. Consequently, we explored the transcriptome of the evolved PAO1 and  $\Delta$ katA colony biofilm and planktonic culture to elucidate the transcriptomic changes leading to antibiotic resistance evolution.

### Methods

PAO1 and  $\Delta$ katA colony biofilm and planktonic cultures were evolved in the presence of 0.1  $\mu$ g/ml ciprofloxacin (1/5 minimal biofilm inhibitory concentrations) or in the absence of antibiotics for six passages. The RNA of evolved populations was sequenced in single end mode on an Illumina HiSeq 2500 device (1 x 50 bp reads).

### Results

The transcriptomic analysis of PAO1 and  $\Delta$ katA evolved populations indicates higher gene expression in CIP-evolved populations than CTRL populations. Similarly, biofilms (both CIP and CTRL) exhibit more expressed genes than planktonic populations, with minimal overlap between biofilm and planktonic conditions. In CIP-biofilm evolved populations, there is an upregulation of the phage region PA0614-PA0722 and SOS-response genes (*recA*, *recN*, *lexA*). The *pfpl* gene, associated with oxidative stress protection, is also upregulated. Conversely, in CIP-planktonic populations, anthranilate degradation genes show upregulation, leading to the upregulation of *catA*, *B*, and *C*, suggesting a biofilm-specific response to ciprofloxacin. Anaerobic respiration genes (*norB*, *norC*, *nirC*, *nirL*, *nirM*, *nirS*, *moeA1*) are upregulated in PAO1 CIP-biofilm evolved populations compared to PAO1 CIP-planktonic and  $\Delta$ katA CIP-biofilm populations. Additionally, *recF* and *mutL* genes exhibit upregulation in  $\Delta$ katA CIP-biofilm evolved populations compared to PAO1 CIP-biofilm evolved populations.

### Conclusion

Overall, this comprehensive analysis enhances our understanding of the intricate transcriptomic changes in Pa populations under ciprofloxacin exposure, shedding light on both shared and population-specific adaptive responses. These findings hold significance for unraveling the dynamics of antibiotic resistance and bacterial evolution in different environments.

### Keywords:

*Pseudomonas aeruginosa*, Biofilms, antibiotic resistance

Invited speaker:

## Resistance to Bacteriophage Therapy

Laurent Debarbieux

*Bacteriophage, Bacterium, Host Institut Pasteur*

Bacteriophages are the most abundant viruses on Earth. They infect bacteria in virtually any place where bacteria thrive (oceans, soils, animals, plants). Because of their capacity to kill bacteria they were proposed for treating bacterial infections in humans as early as 1917. After a short development worldwide, phage therapy was almost abandoned to the beneficence of antibiotics. However, over the years bacteria have managed to resist to multiple antibiotics. Currently, facing the threat of the lack of new antibiotics, the therapeutic potential of bacteriophages is being re-evaluated.

After more than century of research on bacteriophages, we know the main mechanisms taking place during bacterial infection. Conversely, we also learned how bacteria defend themselves against bacteriophages. However, these observations were vastly derived from in vitro experiments performed with a reduced number of model phages.

Metagenomic approaches and the renewed interest on phage therapy have recently fueled the discovery of many new phages. It was then realized that the diversity of interactions between bacteriophages and bacteria is much larger than expected. For instance, hundreds of new putative resistance mechanisms have been identified from bioinformatic analyses and more than a dozen have already been experimentally confirmed.

Using pre-clinical murine models of lung infection and gut colonization we investigated the potential of bacteriophages to infect bacteria in these organs. We found that the efficacy of a single bacteriophage to treat pulmonary infections relies on a synergistic action with the immune response to control the growth of phage-resistant bacteria. By contrast, in the gut, the impact of one or a cocktail of bacteriophages on the levels of bacteria remains moderate, and we were unable to recover phage-resistant clones.

Finally, in line with the report of the first 100 patients who received a compassionate treatment in Belgium, I will discuss the phage-resistance mechanisms observed in vivo in the light of those obtained in vitro.

## Unraveling mechanisms underlying biofilm-induced tolerance in *Pseudomonas aeruginosa* by applying a multi-omics approach

Dr. Janne G. Thöming<sup>1,2</sup>, Dr. Matthias Preusse<sup>3</sup>, Prof. Susanne Häussler<sup>1,2,3,4</sup>

<sup>1</sup>Rigshospitalet, Clinical Microbiology, Copenhagen, Denmark, <sup>2</sup>TWINCORE, Molecular Bacteriology, Hannover, Germany, <sup>3</sup>Helmholtz Centre for Infection Research, Molecular Bacteriology, Braunschweig, Germany, <sup>4</sup>Hannover Medical School, Cluster of Excellence RESIST (EXC 2155), Hannover, Germany

### Background

*Pseudomonas aeruginosa* is a major bacterial pathogen causing persistent infections often associated with biofilm formation. Sessile biofilm bacteria are embedded and protected in a self-produced extracellular matrix and exhibit profound changes in bacterial physiology. Antimicrobial therapy usually fails despite the absence of genotypic resistance, and it is commonly accepted that biofilm-grown bacteria are 10 to 1,000 times more tolerant than their planktonic counterparts. However, despite the clinical relevance, we are only at the beginning to understand the underlying mechanisms of biofilm recalcitrance.

The aim of this study is to gain new insights into the complex adaptation of *P. aeruginosa* during infection and the development of biofilm-induced tolerance to antimicrobial treatment.

### Methods

We screened a large collection of 350 clinical *P. aeruginosa* isolates for their susceptibility profiles under biofilm conditions against ciprofloxacin, tobramycin, and colistin. Based on characteristic patterns of drug-specific killing activity, we classified strains into groups exhibiting high- or low-level biofilm tolerance. Representative strains were subjected to in-depth multi-omics analyses (RNA-seq, metabolomics and Tn-seq) to uncover the cellular mechanisms that drive the observed differences in biofilm tolerance.

### Results

In this study, we detected increased levels of the TCA intermediates succinate and fumarate as well as elevated levels of pyruvate and lactate in biofilms of highly tolerant isolates, indicating different rates of glucose utilization and non-oxidative fermentation compared to sensitive biofilms. RNA-seq revealed distinct regulatory patterns in genes encoding ATP synthase, suggesting reduced ATP production in biofilms of tolerant bacteria. In addition to an altered energetic state, tolerant biofilms also appeared to have a different redox state. In highly tolerant biofilms, NAD<sup>+</sup> levels and GSSG (oxidized glutathione) were elevated, suggesting an imbalance in the cellular redox state towards oxidized forms. This possibly triggers the induction of the oxidative stress response and increased tolerance towards antibiotics. Additionally, alterations in lipid metabolism hinted at membrane permeability as an additional determining factor for the observed differences in biofilm tolerance.

### Conclusions

These findings provide valuable insights into the multifaceted mechanisms driving biofilm-induced tolerance in *P. aeruginosa*, paving the way for novel targeted strategies to overcome the challenges posed by chronic, biofilm-associated infections.

### Keywords:

*Pseudomonas aeruginosa*, biofilm-tolerance, transcriptomics, metabolomics

## How does *Pseudomonas aeruginosa* Biofilms Cope with Antibiotic Challenge?

Dr Ainelen Piazza<sup>1</sup>, Harshanie Dasanayaka<sup>1</sup>, Prof. Mark A. Webber<sup>2</sup>, Dr Freya Harrison<sup>3</sup>, Dr Jacob G. Malone<sup>1</sup>

<sup>1</sup>John Innes Centre, Norwich Research Park, United Kingdom, <sup>2</sup>Quadram Institute Bioscience, Norwich Research Park, United Kingdom, <sup>3</sup>School of Life Sciences, The University of Warwick, Coventry, United Kingdom

### Background

The ESKAPE group bacterium *Pseudomonas aeruginosa* poses a significant threat as a multidrug-resistant human pathogen. Chronic infections by this bacterium often involve biofilm formation, conferring resistance to antimicrobial agents and complicating treatment strategies. Within biofilms, uncharacterized genes may play pivotal roles, suggesting novel factors in the biofilm mode of growth. This study focuses on understanding the role of the uncharacterized gene PA3049 in biofilm-associated antibiotic tolerance.

### Methods

Employing a multidisciplinary approach, we characterised PA3049 and its importance for *P. aeruginosa* biofilm formation. We first utilized bioinformatics to investigate the distribution of PA3049 across bacterial genomes. Next, we assessed PA3049 impacts on infection using the ex vivo pig lung (EVPL) and Synthetic chronic wound (SCW) models, where we examined biofilm formation, infection clearance and antimicrobial resistance. Finally, we explored the mechanism of action of the PA3049 protein in *P. aeruginosa* using a combination of molecular microbiology and biochemistry techniques.

### Results

PA3049, identified as a hydrophilin protein, is annotated as a homolog of the *Escherichia coli* ribosome modulator factor (RMF), crucial for ribosome hibernation in *E. coli*. However, PA3049 does not fulfil a similar function in *P. aeruginosa*. In the context of *P. aeruginosa* biofilms, PA3049 emerged as a player in withstanding antimicrobial challenges both in vitro and in the EVPL and SCW models. Deletion of PA3049 resulted in reduced pyocyanin production and altered abundance of enzymes controlling denitrification, pyoverdine, and hydrogen cyanide biosynthesis. PA3049 directly interacts with two kinases implicated in antibiotic tolerance, inactivating them. These interactions explain most of the phenotypes associated with BatR and present a compelling model for the in vivo mechanism of action of this small biofilm regulator.

### Conclusions

Renamed as the Biofilm Antibiotic Tolerance Regulator (BatR), PA3049 is a key player in *P. aeruginosa* biofilm maintenance and antimicrobial tolerance. These findings contribute to understanding the complex bacterial lifestyle in biofilms, shedding light on a previously uncharacterized gene with significant implications for combating multidrug-resistant infections.

### Keywords:

Hydrophilins, Antibiotic tolerance, Biofilm, Infection

## MODEL SYSTEMS

Invited speaker:

### Pharmacokinetic and -dynamics of biofilm associated infection: How experimental and computational models can inform dose optimization

Coen van Hasselt

Understanding pharmacokinetic (PK) and pharmacodynamic (PD) relationships of antimicrobials is essential for rational dose selection and optimization for treatment of bacterial infections. The use of experimental infection models and mathematical models are both essential tools to support this process. In drug development or further dose optimization studies of antimicrobials, typically, the biofilm state is often not, or not sufficiently considered. This presentation will introduce key concepts in PK/PD of antimicrobials and how these concepts and approaches can be applied to biofilm-associated infections. Key features of experimental biofilm infection models are then further discussed from the perspective of PK/PD characterization, and how results of such models can be coupled to clinical PK models to translate experimental findings to clinical treatment strategies.

## The role of polymicrobial biofilms in urinary tract infections (UTIs) using a urine tolerant human urothelium organoid model (3D-UHU)

Mr. Ramon Garcia Maset, Richard Leung, Dr Ian White, Professor Claire Smith, Professor Jennifer Rohn

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Urinary tract infections (UTIs) are among the most frequent types of infection (Öztürk and Murt, 2020). The global incidence of UTIs in 2019 reached 0.4 billion cases, with an associated increased mortality (Zeng, 2022). UTIs are also a major driver of one of the century's biggest challenge: antimicrobial resistance (AMR). The role of biofilm formation in UTI is poorly understood and is mainly focused on catheter infections; in other settings, however, biofilm formation is associated with chronic infections and results in high resistance to and tolerance of antibiotics (Bjarnsholt, 2022). The possibility of biofilm-like communities on the bladder surface of non-catheterised UTI patients might explain the failure of treatment and the high recurrence rates. As a related issue, polymicrobial biofilms are present in almost all types of human chronic infections. However, polymicrobial infection is challenging to study in vitro and tend to be understudied, especially in the UTI field (Armbruster, 2021).

Herein, we investigated the biofilm-forming capabilities of diverse uropathogens in human urine-containing media instead of standard culture media. Additionally, we evaluated the fitness of paired combinations of the most prevalent UTI pathogens in human urine vs standard media. Unexpectedly, certain pathogen combinations coexist in standard media but exhibit competitive dynamics in urine-containing media. Furthermore, we explored the biofilm formation of uropathogens as a single species and as dual combinations, employing a state-of-the-art 3D urine-tolerant human urothelial (3D-UHU) microtissue model. Our study presents the first example of dual-species and three-species biofilms cultivated in the context of a complex human urothelium. These findings enhance our comprehension of single-species and polymicrobial biofilm dynamics in UTIs and underscore the significance of media selection and microenvironment in discerning pathogen interactions.

### **Keywords:**

Polymicrobial, urinary tract infection, organoids

## In vitro characterization of *Trichophyton rubrum* biofilm and infection in three-dimensional keratinocytes culture

undergraduated student Matheus Bordoy Mendonça<sup>1</sup>, undergraduated student Bruno Bulgarelli Adorno Arantes<sup>1</sup>, doctoral student Ana Karla Lima Freire Cabral<sup>1,2</sup>, PhD Kaila Petronila Medina-Alarcón<sup>1</sup>, doctoral student Kelvin Sousa dos Santos<sup>1</sup>, PhD Ligia Fernandes, PhD Ana Marisa Fusco-Almeida<sup>1</sup>, PhD Maria Giannini<sup>1</sup>

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### Background

*Trichophyton rubrum* is a keratinophilic fungus and is known for causing dermatophytoses. These infections frequently become chronic and complex to treat, mainly onychomycosis. The ability to form biofilms is a virulence factor that could contribute to this chronicity and treatment difficulty. Furthermore, its anthropophilic nature restricts the studies about these in vivo biofilm formations. Therefore, this study aimed to demonstrate biofilm formation in vitro and develop a three-dimensional culture of keratinocytes to study the host-parasite interaction.

### Methods

*T. rubrum* was cultured in planktonic form for seven days, after which the biofilm was formed. Fungal biomass and metabolic activity were assessed using crystal violet and tetrazolium salt reduction (XTT) tests. The spheroids were formed using human epidermal keratinocytes (HaCat) in two concentrations over 96 hours at 37°C and 5% CO<sub>2</sub>, and their viability was evaluated by resazurin assay and image analyses using the software ImageJ. Upon keratinocyte spheroids formation, the medium was removed, and a suspension was added at 1x10<sup>6</sup> conidia/mL in DMEM medium. The interaction between the biofilm and the spheroids was subsequently analyzed utilizing fluorescence microscopy using two fluorochromes.

### Results

*T. rubrum* biofilm matured at 72 hours, with significantly high metabolic activity and biomass formation. The keratinocyte spheroids were fully formed at 96 hours, and the cells maintained their viability for seven days. Both the cell concentrations presented similar sphericity indexes, but the higher concentration had a larger radius. After the spheroid's infection, it was possible to observe that fungal structures had developed around and over the spheroid in fluorescence microscopy. The ideal concentration for forming HaCat spheroids was defined, and the markers elucidated the interaction between the tridimensional model and *T. rubrum* biofilm.

Conclusion. This study can potentially clarify new perspectives about dermatophyte biofilms, including elaborating possible markers as new targets for in vivo research and anti-biofilm therapy. Funding: FAPESP, process 2022/15456-9 and 2023/035556-1.

### Keywords:

*T. rubrum*, biofilm, spheroids

Invited speaker:

## Animal implant biofilm models

Louise Kruse Jensen

Animal models are essential for studying biofilms. To truly understand the complex interaction between implant material, immune response, bacteria, and host tissue, along with comorbidities, animal models are necessary. However, it's important to acknowledge that an animal model is a simplification of the truth. So, how can we minimize this simplification? How can we make animal models as clinically relevant as possible for studying implant-related biofilm infections? These questions will be addressed in the presentation.

Additionally, the presentation will cover the development, characterization, and application of a porcine model for implant-related osteomyelitis as an example of studying implant biofilm infections in vivo. It will also highlight various bacterial immune evasion strategies, including biofilm, when residing by bacteria in bone tissue and discuss how such pathophysiological knowledge should be used to guide development of new treatment strategies. The examination of local tissue using pathological methods will be a central focus.

## Modeling Biofilm Infection in the Murine Sinonasal Cavity

Emily Vanderpool<sup>1,2</sup>, Kendra Rumbaugh<sup>1,2</sup>

<sup>1</sup>Department of Surgery and Burn Center of Research Excellence, Texas Tech University Health Sciences Center, Lubbock, United States, <sup>2</sup>Department of Immunology and Molecular Microbiology, Texas Tech University Health Sciences Center, Lubbock, United States

### Background and Aims

Models of biofilm infection that recapitulate disease conditions are critical to better understand the sequela in many biofilm-associated infections. One of these conditions, chronic rhinosinusitis (CRS), affects 10-15% of the adult population worldwide. CRS has been one of the leading reasons for primary and ENT clinic visits, as well as one of the top diagnoses associated with antibiotic prescription despite clinical evidence of efficacy.

Biofilm has been confirmed as a player in CRS, but its specific role as a driver, contributor, or consequence has not been clearly defined. Thus, we set out to develop a novel model of biofilm infection in the sinonasal cavity of mice.

### Methods

Using commonly identified CRS bacterial species, *Pseudomonas aeruginosa* (PA) and *Staphylococcus aureus* (SA), we tested for the persistence of inoculated bacteria and evidence of biofilm formation within the murine sinonasal cavity under various conditions. In accordance with proposed hypotheses regarding biofilm formation in CRS, we tested preceding inflammation, mucus accumulation, interference with  $\beta$ -defensins, and alterations to normal flora. Additionally, we tested inoculations with SA and PA in planktonic form, in pre-formed biofilms, and dispersed from biofilms, as well as planktonic bacteria embedded in various matrices. For each condition, we quantified host-specific indications of CRS, including inflammatory cytokines and sinonasal inflammation.

### Results

We found that at 5 days post-inoculation, bacterial load from the inoculated strains remains moderate ( $\leq 10^5$  colony forming units/gram of tissue) under almost all of the conditions tested. However, embedding bacteria in matrices such as alginate increased bacterial load to  $10^8$  colony forming units/gram of tissue, but resulted in lower respiratory tract infection. We plan to perform imaging on these nasal samples for evidence of biofilm formation, as well as to compare serum cytokine profiles and histological changes.

### Conclusions

Our results may indicate that historically accepted hypotheses regarding conditions for the development of biofilm infection in CRS are incomplete, warranting further investigation. Finally, our results underscore the need for continued development of disease-relevant models to characterize and treat biofilm-associated infections.

### Keywords:

chronic rhinosinusitis, in vivo models

## The role of Salmonella biofilm formation in the porcine gut

Ir. Fran Gilis<sup>1,2</sup>, Thijs Van Leemputte<sup>1</sup>, Dr. ir. Josefien van Landuyt<sup>2</sup>, Dr. ir. Bram Lories<sup>1</sup>, Prof. Dr. ir. Tom Van de Wiele<sup>2</sup>, Prof. Dr. ir. Hans Steenackers<sup>1</sup>

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<sup>2</sup>Center for Microbial Ecology and Technology, Faculty of Bioscience Engineering, UGent, Gent, Belgium

### Background

Chronic Salmonella infections in pigs are prevalent, but asymptomatic. They are therefore difficult to diagnose and are a concern for the food safety when undetected. While biofilm formation is crucial for Salmonella's persistence outside of the host, the role of in vivo biofilm formation in these chronic infections remains unclear. Therefore, this research aims to elucidate to what extent biofilm formation contributes to gut colonization in pigs.

### Methods

Biofilm formation capacity of a Salmonella lab strain (American Type Culture Collection 14028) and Salmonella natural isolates, obtained from pig farms, was determined in vitro by quantifying the number of cells adhering to the surface as well as the biofilm matrix production level. Subsequently, the role of biofilm formation of three isolates exhibiting high in vitro biofilm production was evaluated using the simulator of the mucosal pig intestinal microbial ecosystem (M-SPIME). This model consists of sequential compartments that mimic the different parts of the gastrointestinal tract by adapting the environmental conditions. Initially, a static, single-compartment adaptation of the M-SPIME was performed, where different conditions with increasing complexity were tested. To elucidate the role of biofilm formation, the (I) survival and growth throughout the gastrointestinal tract, (II) mucus colonization and (III) competition with the gut microbiota was compared between the wildtype and biofilm-deficient deletion mutants.

### Results

Overall, the natural isolates were generally weaker biofilm formers in vitro compared to the lab strain. The preliminary results in the static single-compartment assay indicate a strain-dependent impact of biofilm formation on survival of the harsh conditions in the gut, but a minimal role for biofilm formation in the mucus colonization and competition with the gut microbiota. Moreover, we observed that Salmonella more easily survived the harsh environment of the porcine gut and the competition with gut microbiota when residing in the mucus, suggesting an important protective role of mucus during gut colonization.

### Conclusion

Our initial findings indicate that both the mucus colonization ability and biofilm formation capacity can provide protection to Salmonella across the simulated porcine gut. This will be further confirmed using the dynamic, multi-compartment model and an in vivo pig trial.

### Keywords:

Salmonella, porcine gut simulator

Saturday 29 June

## CORRELATION OR CAUSALITY?

Invited speaker:

### Correlation or causality? Periodontitis and Cardiovascular disease

David Herrera

Periodontitis, an inflammatory condition of infectious nature which inflamed and eventually destroys the supporting tissues of teeth, is an important public health problem due to its high prevalence: it is the most common chronic inflammatory non-communicable disease (NCD) of humans. It is also a major public health burden due to its associated morbidity, leading to disability due to impaired masticatory function, speech and aesthetics, and to edentulism. It is a source of social inequality, significantly impairs quality of life, has a negative impact on general health, and is associated with significant dental and medical care costs.

Periodontitis has been associated with a broad range of systemic conditions, including diabetes, cardiovascular diseases, respiratory diseases, adverse pregnancy outcomes, rheumatoid arthritis, different types of dementia including Alzheimer`s disease, or some cancers.

Cardiovascular diseases (CVDs) represent a diverse group of different conditions, including ischemic heart disease, stroke, hypertension, rheumatic heart disease, cardio-myopathies and atrial fibrillation. CVDs are the leading cause of mortality worldwide, representing 32% of all global deaths and 45% of NCD-related mortality.

Evidence derived from epidemiological studies indicate that periodontitis increases the risk of different CVDs: coronary heart disease (i.e. coronary artery disease, myocardial infarction), cerebrovascular disease (i.e. ischaemic stroke), or peripheral artery disease. It has also been associated with higher mortality rates due to coronary heart disease and cerebrovascular disease. Conversely, there is no direct evidence on the benefits of periodontal treatment on cardiovascular risk, although there is clear evidence on the effect of periodontal therapy on surrogate markers of cardiovascular diseases, arterial blood pressure and stiffness, and subclinical acute cardiovascular diseases markers.

Although different biological explanations have been proposed, frequent bacteraemia from periodontal pockets in periodontitis patients and an increase in systemic inflammation markers, provide the biological plausibility for the associations between periodontitis and CVDs.

Therefore, exploring the role of oral biofilms and, specifically, subgingival biofilms, is crucial to understand the association between periodontitis and different CVDs, including the role of oral and periodontal bacteria in atheroma plaque formation and/or rupture.

## Association of Biofilm and Microbial Metrics with Healing Rate in Venous Leg Ulcers

Dr. Phil Stewart<sup>1</sup>, Junglyun Kim<sup>2</sup>, Garth James<sup>1</sup>, Fan Yi<sup>3</sup>, Joyce Stechmiller<sup>4</sup>, Michael Weaver<sup>4</sup>, Debra Kelly<sup>4</sup>, Daniel Gibson<sup>5</sup>, Gregory Schultz<sup>4</sup>, Debra Lyon<sup>4</sup>

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### Background

The presence of microbial biofilms in many human chronic wounds led to the hypothesis that the amount of biofilm delays healing of these wounds. This conjecture has never been clinically and quantitatively tested in a significant human population. The overall objective of this study was to report associations between the amount of biofilm and other microbial metrics and the rate of healing of a defined subset of human chronic wounds.

### Methods

The hypothesis was tested in a population of 117 older individuals with venous leg ulcers who were receiving standardized therapy, including frequent debridement. Debridement specimens were analyzed for the amount of bacterial biomass by two independent methods: a microscopic approach that scored the relative size and number of bacterial aggregates, interpreted as a biofilm metric, and conventional enumeration by agar plating for viable bacteria. The plating protocol yielded three distinct values: the total viable bacterial count, bleach-tolerant bacteria, and bleach-susceptible bacteria. Wound healing rates over an 8-week observation period were calculated as the rate of decrease of the equivalent diameter of the wound.

### Results

There was no statistically significant association between wound healing and the biofilm metric in any of the three analyses performed ( $p \geq 0.15$ ). In all three statistical tests, wound healing was associated with the number of bleach-susceptible bacteria ( $p \leq 0.004$ ); wounds that harbored more bleach-susceptible bacteria healed more slowly. Wound healing rates were not associated with the number of bleach-tolerant bacteria in debridement specimens.

### Conclusions

This study did not find evidence for a causal relationship between the amount of biofilm in a wound and the clinically quantified wound healing rate. As the number of bleach-susceptible bacteria increased, wound healing rates decreased. A refinement of the model of chronic wound infection pathogenesis is proposed in which the contribution of the biofilm (alternatively, the metabolically inactive population) is to serve as a nidus for recurrence of the infection whereas impaired healing results from the outgrowth of planktonic (alternatively, metabolically active) bacteria.

### Keywords:

wound, CVLU, healing, biofilm

## Wound microbiome interactions and healing: a retrospective and in vivo approach

Rebecca Gabriliska<sup>1</sup>, Dr. Joseph Wolcott<sup>2</sup>, Dr. Kendra Rumbaugh<sup>1</sup>

<sup>1</sup>Department of Surgery, Texas Tech University Health Sciences Center, Lubbock, United States, <sup>2</sup>Southwest Regional Wound Care Center, Lubbock, United States

### Background

Nonhealing chronic wounds are a burden for patients and healthcare providers globally. Chronic wounds are notoriously polymicrobial with a rich wound microbiome in biofilms. Although these biofilms have been shown to contribute to recalcitrant infections, it has also been demonstrated that the members of the wound microbiome can behave as commensals or pathogens. This study aims to better understand the dynamics of microbe-microbe interactions within the wound microbiome. Utilizing patient records and a mouse wound model, we focused on the most implicated and prevalent bacteria *Staphylococcus aureus* as a fulcrum to investigate microbial interactions. As *Staphylococci* are found in both healing and non-healing wounds, we hypothesized that *S. aureus* behaves as a commensal in wounds when paired with known skin microbiota and a pathogen when paired with non-commensal bacteria.

### Methods

Patient retrospective chart review of over 1,300 chronic wounds was used to investigate correlations between wound healing and the wound microbiome. Observations from human wound microbiomes were subsequently translated to an in vivo polymicrobial murine wound model to assess the impact of microbe-microbe interactions on wound closure.

### Results

Microbiome diversity matrices between healing and nonhealing wounds were unremarkable. Across all wounds, community richness was comparable to previously reported wound studies, including *S. aureus*, *S. epidermidis*, *C. striatum*, *E. coli*, *F. magna*, and *P. aeruginosa*. In wounds with an unremarkable course (i.e. non-repeated NGS diagnostics), *S. aureus* was found with high abundance of skin commensals, despite prevalence of *S. aureus* in all wounds. Once these communities were translated onto murine wounds, we further observed their differential contribution to healing inhibition was dependent on the microbes in the wound.

### Conclusions

With the growing number of microbiome studies, additional research is needed to translate the associations between microbiome matrices and patient outcomes into causal relationships. These results strive to inspire future studies to refine diagnostics and optimize disease management.

### Keywords:

Chronic wounds, microbiome, interactions

## Invited speaker:

### Commensal biofilm turns pathogenic: a link from gut inflammation to cancer

Jean-Paul MOTTA, PhD

*INSERM, Institute of Digestive Health Research, Toulouse, France; University of Toulouse, Toulouse, France*

Biofilms are complex microbial communities forming a “biological film” on surfaces. In natural habitats, microbes typically form biofilm communities, embedded in a biopolymer matrix, interacting with surfaces, and engaged in collective behaviors. Mucosal surfaces in human body, such as those in the intestine are densely colonized by microbial communities as biofilms.

Current knowledge on how microbes influence host physiology and pathology largely comes from studies on free-living (planktonic) microbes. Therefore, understanding how in vivo mucosal biofilms impact gut physiology is of significant interest. This lecture also aims to move beyond the traditional view of biofilms as solely harmful and challenge the notion that gut microbes act as isolated entities in host-microbial interactions. In addition to discussing taxonomic features, I will explore how phenotypic characteristics of gut biofilms may serve as fine indicators of microbiome behavior and how it may influence gut health. I will present experimental evidence of the inflammatory response to altered biofilm communities using collections of human-derived biofilms from both chronically inflamed diseases and aging populations. This talk will also present studies describing various microbial microhabitats across intestinal mucosal surfaces, thereby enhancing our understanding of the contributions of microbial biogeography to intestinal health. Finally, given that chronic inflammation is a recognized factor in tumorigenesis, I will discuss how mucosal biofilms contribute to understanding the microbial impact on gut tumorigenesis.

## Temporal Evolution and Functional Characteristics of Biofilm Formation on Reverse Osmosis Membranes in Tertiary Effluent Desalination

Prof Osnat Gillor<sup>1</sup>

<sup>1</sup>*Ben Gurion University Of The Negev, , Israel*

In tertiary effluent desalination, biofouling on membranes poses significant challenges, including increased downtime, reduced lifetime, higher energy consumption, and loss of permeability. While the diversity and composition of the biofouling layer have been extensively studied, little is known about the succession of the bacteria in the growing biofilms or their expression and functions. To address this, we employed a benchtop reverse osmosis (RO) system to desalinate synthetic tertiary effluent supplemented with diluted sludge. We monitored system flux to assess biofouling layer development and collected RO biofilm samples at early and mature stages (after 48, 72, 96, and 120 hours). Samples were visualized using scanning electron microscopy, 16S rRNA genes were sequenced, in addition to the biofilms transcriptomes. DNA-stable isotope probing (SIP) was used to identify growing taxa by spiking the system with labeled glucose in early and late biofouling stages. Additionally, quartz crystal microbalance with dissipation (Q-CMD) characterized extracellular polymeric substances (EPS) affinity to membrane-mimetic sensors. We thus evaluated the viscoelasticity of the Ex-Situ EPS layer formed on the sensor. Our results showed different active bacterial taxa across five taxonomic classes assembled on the RO membrane, with composition shifts. Proteobacteria were predominant in the early stages, alongside Bacteroidota. Actinobacteria become more prevalent in mature biofilms, aiding in community preservation and biofilm structure maintenance. However, regardless of composition, biofilm maturation led to similar gene families expression associated with temporal kinetics of EPS composition, validated by the adhesion, and viscoelastic properties. This study provides insights into temporal dynamics and functional traits of RO biofouling, shedding light on mechanisms driving membrane deterioration during water purification processes.

### **Keywords:**

DNA-SIP. RNA-Seq, biofouling, desalination

## When a Paradiem Biofilm Infection and a Revolutionary Treatment Collide: Cystic Fibrosis (CF) Lung Infections in the Age of Modular Therapy

Professor Pradeep Singh<sup>1</sup>

<sup>1</sup>University of Washington, Seattle, United States

### Background

The *Pseudomonas aeruginosa* (Pa) lung infections that afflict people with CF are considered a paradiem biofilm disease. Drugs called modulators improve the CF host defense defect, and elxacaftor/tezacaftor/ivacaftor (ETI) is the most effective modulator in clinical use. Understanding how CF infections respond after lung host defenses are improved has important implications for health of people with CF and biofilm research in this area.

### Methods

We present 2 studies that advance understanding of ETI's effects on CF lung infections and inflammation. The first (PROMISE-study), examined 236 subjects and used sputum culture, PCR and sequencing. The second (BAL-study), studied 9 subjects at high resolution using bronchoscopy to sample each subject's 2 most and 3 least-damaged lung regions before and 1.5 years after ETI, with comprehensive measurements of infection, inflammation and metabolic parameters on regional washings.

### Results

Promise found that average sputum density of Pa and other CF pathogens decreased ~100-fold after 1 month, with little further change through 3 yrs. Notably, most participants remained culture-positive. In addition, the pathogens present before treatment generally remained detectable by PCR even in those becoming culture-negative. Sequencing found increased sputum bacterial diversity and bacterial composition shifts. However, these changes were caused by treatment-induced decreases in traditional CF pathogens.

The bronchoscopy study found that subjects remaining infected continued to harbor Pa in high and low-damage lung segments, contradicting the idea that infection persists only in highly-damage lung regions. In addition, while inflammation generally decreased, the coefficient of determination (i.e. the R<sup>2</sup> value) between Pa density and inflammatory makers increased after ETI, indicating that Pa density better explained inflammation post-ETI as compared to pre-ETI. Moreover, the slope of the relationship between Pa density and inflammatory makers increased after ETI, indicating that ETI did not reduce the extent of inflammation induced by Pa on a per-cell basis.

### Conclusions

Treatment with the most effective CFTR modulator reduced traditional CF pathogens in sputum, but most participants remained infected. Invasive sampling showed that Pa remains widespread throughout the lungs of infected subjects and that infection continues to induce lung-damaging inflammation.

### Keywords:

Cystic Fibrosis; Infection; Modulators, Inflammation

## IMAGING AND/OR NOVEL TECHNICS IN BIOFILM RESEARCH?

Invited speaker:

### Revealing biofilm functions by quantitative biofilm image analysis at cellular resolution

Knut Drescher

*Affiliation: Biozentrum, University of Basel, 4056 Basel, Switzerland*

In nature, bacteria mostly live in surface-attached communities termed biofilms, in which cells are attached to each other through an extracellular matrix. In this presentation, I will first introduce microscopy and image processing techniques that enable us to monitor all individual cells in living biofilms. Based on these techniques, I will then show how we can quantify biofilm properties in space and time, which revealed new functions of biofilms. The specific biofilm function I will focus on in the presentation arises during the interaction with immune cells. I will show that for the human pathogen *Vibrio cholerae*, biofilm formation is not only a protective trait but also an aggressive trait to collectively predate different immune cells. We find that *V. cholerae* forms biofilms on the eukaryotic cell surface using an extracellular matrix comprising primarily mannose-sensitive hemagglutinin pili, toxin-coregulated pili, and the secreted colonization factor TcpF, which differs from the matrix composition of biofilms on other surfaces. These biofilms encase immune cells and establish a high local concentration of a secreted hemolysin to kill the immune cells before the biofilms disperse in a c-di-GMP-dependent manner. Together, these results uncover how bacteria employ biofilm formation as a multicellular strategy to invert the typical relationship between human immune cells as the hunters and bacteria as the hunted.

## Deep Learning and 3D Holographic Microscopy to assess the early bacterial adhesion's dynamics under shear rate in microfluidic systems

Miss. Lucie Klopffer<sup>1,2</sup>, Dr Laurence Mathieu<sup>1,4</sup>, Pr Cédric Pradalier<sup>3</sup>, Mr Simon Becker<sup>2</sup>, Dr Nicolas Louvet<sup>2</sup>

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<sup>3</sup>GeorgiaTech Europe IRL 2958, Metz, France, <sup>4</sup>EPHE PSL, UMR 7564 CNRS, Nancy, France

Pioneer bacterial adhesion events play a crucial role in biofilm formation and need further understanding to appreciate bacterial colonization and consider appropriate control strategies. In this context, our study aims at implementing 2 complementary approaches based on Deep Learning and Holographic Digital Microscopy. Our objective is to monitor in 3D space and time the dynamics of wall transfer and adhesion of a model bacteria in a home-made microfluidic system under different hydrodynamics (from 0 to 1250 s<sup>-1</sup>). This device is mounted under an epifluorescence microscope equipped with a CCD camera and fed 2 hours with a *Shewanella oneidensis* MR-1 suspension (stationary phase; 10<sup>7</sup> cells/mL). From images recorded, Deep Learning analysis allows massive data and information extractions of the bacteria dynamics at the wall i.e. their attachment/detachment frequencies, residence time, orientation and movement. The Holographic Digital Microscopy technic enables to access bacteria dynamics in 3 dimensions and appreciate bacteria transfer mechanisms at the wall, trajectories or speed in the bulk and near the wall.

Our preliminary Holographic Microscopy results showed that about 10% of the bacteria in the inoculum are initially motile with an average speed of 40 μm/s. While bacteria trajectories are relatively straight in the bulk, close to the wall we observe that bacteria spend a long time to swim with complex trajectories. Moreover, Deep Learning analysis revealed that the orientation of the pioneering adhered bacteria at the wall under static conditions follow random distribution, while bacteria subjected to hydrodynamic constrains were mostly oriented parallel to the flow. Quite unexpectedly, we also observed that most of the adhered bacteria presented a very short residence time at the wall, i.e. < 10 seconds, which could question the capability for further colonization. To this end, we have characterized the attachment / detachment frequencies and measured the increased surface colonization over time. The hydrodynamics significantly impacted the bacterial colonization kinetics with notably better colonization speed and level obtained for wall shear rate close to 125 s<sup>-1</sup>. These two approaches help to better characterize the behavior and dynamics at the very early steps of adhesion of pioneer bacteria at the wall.

### **Keywords:**

Deep-Learning; Holographic-Microscopy; early adhesion; Hydrodynamics

## Microdialysis sampling for monitoring the secretome between macrophages and biofilm co-cultures

Alda Alexa Diaz, Victoria Hunter, Patrick Pysz, Sage Bentlage, Gillian Waters, Julie Stenken<sup>1</sup>

<sup>1</sup>University Of Arkansas, Fayetteville, United States

### Background

Bacteria communicate chemically via unique chemical signals, called quorum sensing chemicals, associated with their species and include various lactones, quinolones as well as cyclic peptides. For immune system cells including macrophages, a subclass of chemical communication involves cytokine signaling proteins. Macrophages also secrete cathelicidin, h-CAP-18, which is a pro-peptide for LL-37, an antimicrobial peptide. While it is known that immune-biofilm interactions involve quorum sensing, the mechanisms and temporal profiles of these processes are not fully understood. Having the ability to locally monitor these chemical signals within a biological system would be of significant benefit. This work aimed to create a microdialysis-sampling based system for secretome temporal collection from biofilm/macrophage co-cultures.

### Methods

CMA-20 microdialysis probes (500  $\mu\text{m}$  external diameter) with 10 mm polyethersulfone (PES) membranes were procured from Harvard Apparatus (Cambridge, MA, USA). Bacteria (*Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Staphylococcus epidermidis*) were obtained from the American Type Culture Collection (ATCC). THP-1 cells were a gift from Dr. Steven Wood, US FDA, Silver Spring, MD, USA. Quorum sensing chemicals were analyzed by using UPLC-MS/MS. A separate LC-MS/MS method was used to quantify LL-37. Chemokine, MCP-1, was quantified using ELISA (BD OptEIA). Special 3D printed microdialysis probe holders were created to place into cell culture flasks. Homebuilt 3D printed microfluidic pumps were used to perfuse microdialysis probes at  $\mu\text{L}/\text{min}$  flow rates within a cell culture incubator.

### Results and Conclusions

A variety of quorum sensing chemicals (acyl lactones, acyl oxo lactones and quinolones) were quantified from *P. aeruginosa* biofilm/macrophage co-cultures with the 3-oxo-C12-AHL producing the highest concentration reaching  $620 \pm 200$  ng/mL,  $n=5$ , at 10 hrs. *S. aureus* produced AIP-1 to a level of  $0.9 \pm 0.1$  ng/mL over a period of 4 to 12 hours. All three bacterial species (*P. aeruginosa*, *S. aureus*, and *S. epidermidis*) induced the release of LL-37 from THP-1-based macrophages at different time points over a 24 hr period. Similarly, all three bacterial species elicited varying cytokine (MCP-1/CCL2) responses over a 24 hr period. These results demonstrate that we have the capability to monitor bi-directional cellular secretome dynamics within a biofilm/macrophage co-culture system under varying conditions.

### Keywords:

macrophage, microdialysis, quorum sensing, LC-MS/MS

## Invited speaker:

# Highly multiplexed spatial mapping of microbial species and mobile elements in oral biofilms

Iwijn De Vlaminck

*Affiliation: Nancy E. and Peter C. Meinig School of Biomedical Engineering*

Spatial mapping of the complex biogeography of oral biofilms poses a major challenge because of the high density and rich diversity of species in these microbiomes (1). In this talk, I will introduce high-phylogenetic-resolution microbiome mapping by fluorescence in situ hybridization (HiPR-FISH), a technology that uses binary encoding, spectral imaging and decoding based on machine learning to create micrometer-scale maps of the locations and identities of hundreds of microbial species in complex communities (2). I will discuss application of this methodology to study the longitudinal stability of spatial architectures in the human oral plaque microbiome. In addition, I will present measurements of the spatial arrangement of bacteria in dental implant biofilms from patients with healthy implants or peri-implantitis, an oral microbiome-associated inflammatory disease. I will report distinct spatial architecture associated with dysbiosis, indicating that spatial structure could be used as a biomarker for community stability. Last, I will discuss a novel method which combines HiPR-FISH with single molecule DNA Fluorescence In Situ Hybridization (FISH) to enable simultaneous visualization of Mobile Genetic Elements (MGEs) and bacterial taxa (3). We used this approach to study the spatial spread of bacteriophage and antimicrobial resistance (AMR) plasmids in oral biofilms, and to identify their host taxa. The data suggest spatial heterogeneity in bacterial taxa results in heterogeneous MGE distribution within the community, with MGE clusters resulting from horizontal gene transfer hotspots or expansion of MGE-carrying strains. We hope this approach can help advance the study of AMR and phage ecology in biofilms.

1. Shi H, Grodner B, De Vlaminck I. 2021. *Recent advances in tools to map the microbiome.*

*Current Opinion in Biomedical Engineering* 19:100289.

2. Shi H, Shi Q, Grodner B, Zipfel W, Brito I, De Vlaminck I. 2020. *Highly Multiplexed Spatial*

*Mapping of Microbial Communities.* *Nature* 588:676–681.

3. Grodner B, Shi H, Farchione O, Vill AC, Ntekas I, Diebold PJ, Zipfel WR, Brito IL, De

*Vlaminck I.* 2023. *Spatial Mapping of Mobile Genetic Elements and their Cognate Hosts in*

*Complex Microbiomes.* *bioRxiv* 2023.06.09.544291.

## Extracellular DNA in *Staphylococcus epidermidis* biofilms form a web of DNase-resistant G-quadruplex structures with catalytic activity

Dr. Gabriel Antonio Minero<sup>1</sup>, Dr. Line Mørkholdt Lund<sup>1</sup>, Dr. Andreas Møllebjerger<sup>1</sup>, Dr. Mikkel Illemann Johansen<sup>2</sup>, Dr. Nis Pedersen Jørgensen<sup>2</sup>, Prof. Dr. Daniel Otzen<sup>1</sup>, Prof. Dr. Victoria Birkedal<sup>1</sup>, Prof. Dr. Rikke Meyer<sup>1</sup>

<sup>1</sup>Aarhus University, Gustav Wieds Vej 14, Building 1590, 8000 Aarhus, Denmark, <sup>2</sup>Aarhus University Hospital, Palle Juul-Jensens Blvd. 99, 8200 Aarhus, Denmark

Extracellular DNA (eDNA) is a major matrix component of many biofilms, but it appears to become resistant to enzymatic digestion as the biofilm matures. This can be due to formation of non-canonical structures such as Z-DNA, G-quadruplex (GQ), and DNA:RNA hybrids. The aim of this study was to identify environmental and bacteria-driven factors that promote GQ and Z-DNA formation in the extracellular matrix of *Staphylococcus epidermidis* biofilms, and to detect their susceptibility to nucleases.

The structures were studied *in vitro* in *S. epidermidis* (WT,  $\Delta$ icaADBC,  $\Delta$ atIE) biofilms grown in TSB with 200 mM NaCl and 5  $\mu$ M hemin, and *in vivo* in a murine implant-associated osteomyelitis model with *S. aureus*. The biofilm macroscale structure was imaged by optical coherence tomography. B-DNA and non-canonical DNA structures were visualized by confocal laser-scanning microscopy, using immunolabeling (antibodies 1H6, BG4, Z22 and Ab3519) and DNA-binding stains SYTO<sup>TM</sup>60 and TOTO<sup>TM</sup>-1. Fluorescent tyramide and hydrogen peroxide were used for detection of the peroxidase activity from the GQ/hemin DNAzyme.

Polysaccharides, NaCl, hemin and mechanical stress lead to Z-DNA formation while GQ formation only required NaCl and mechanical stress. GQ-, Z- and B-DNA were abundant in *in vitro* and *in vivo* biofilms, and GQ were both associated with the cell surface and in spiderweb-like strings connecting microcolonies. Staining biofilms with SYTO<sup>TM</sup>60 and TOTO<sup>TM</sup>-1 resulted in GQ-specific FRET in the extracellular matrix.

Macroscale web-like matrix structures also appeared when GQ- and Z-DNA formed. In the presence of hemin, GQ formed a peroxidase-like DNAzyme. DNase I removed B-DNA, but large GQ-rich aggregates emerged after DNase treatment, indicating the DNase I resulted in remodeling of the eDNA. In contrast, Micrococcal nuclease was effective in removing both GQ- and B-DNA.

We believe that an important role of hemin in biofilms has been overlooked until now. Our study revealed its role in promoting Z-DNA and formation of a large web-like matrix. We are the first to show hemin/GQ DNAzyme activity in biofilms, and future studies will reveal how bacteria benefit from this activity.

Some of the results from this work are published in *Nucleic Acids Research* (2024) (DOI: 10.1093/nar/gkae034).

### Keywords:

eDNA, CLSM, FRET, peroxidase, nuclease

## Interplay between sRNA RsaE and individual $\alpha$ Phenol Soluble Modulins ( $\alpha$ PSM) toxins: Implications for Biofilm Formation and Structuring in *Staphylococcus aureus*

Miss Mehak Chauhan<sup>1</sup>, Dr Sander Granneman<sup>1</sup>, Dr Ronan Carroll<sup>2</sup>

<sup>1</sup>Centre for Engineering Biology, University Of Edinburgh, , United Kingdom, <sup>2</sup>The Infectious and Tropical Disease Institute, Ohio University , Athens, United States of America

### Background

*Staphylococcus aureus* causes diverse human diseases through the coordinated expression of multiple virulence factors, including Phenol Soluble Modulins (PSMs).  $\alpha$ PSM1-4 are amphipathic  $\alpha$ -helical cytolytic toxins encoded on the  $\alpha$ psm operon and play crucial roles in bacterial survival by facilitating biofilm formation and structuring. Although all four  $\alpha$ PSMs are structurally related, they have distinct functions and are differentially expressed. While it is known that removing the  $\alpha$ psm operon significantly increases biofilms thickness, the relative contributions of individual  $\alpha$ PSMs remains unclear.

We recently discovered that the RsaE non-coding RNA base-pairs with Shine-Dalgarno sequences of  $\alpha$ psm3 and  $\alpha$ psm2, hinting at selective inhibition of the translation of these two toxins which form  $\alpha$ -amyloid seeds for biofilm accumulation. We speculate that RsaE-dependent regulation of PSM $\alpha$  toxin production enhances the stability of *S. aureus* biofilms, implying an important role for this sRNA-mRNA interaction in immune evasion.

### Aim

We are exploring the contribution of RsaE, individual  $\alpha$ PSMs and their interaction in biofilm formation and structuring.

### Methods

We generated RsaE and  $\alpha$ psm operon deletion mutants along with strains where start codons in individual  $\alpha$ PSMs were mutated. Three techniques were utilised to investigate how these mutations impacted biofilm formation. For each mutant, three technical and three biological replicates were cultivated at three time points (24h, 48h, and 72h). Crystal violet assay was used to quantify total biomass. A combination of fluorescent dyes to stain bacteria, protein, and polysaccharides, was used to study changes in spatial distribution of extra-polymeric substances (EPS). Cryo-SEM analysis was conducted to examine differences in biofilm architecture.

### Result

Clear differences were observed in the total biomass of each mutant. The absence of RsaE,  $\alpha$ 2, and  $\alpha$ 3 led to dispersion of biofilm after 48 hours. Conversely,  $\alpha$ 1 and  $\alpha$ 4 mutants produced much thicker biofilms with more PNAG at 72 hours.

### Conclusion

These results support our hypothesis regarding the differential roles played by individual  $\alpha$ PSMs in the formation and structuring of biofilms. Our data also suggests that PSM  $\alpha$ 1 and  $\alpha$ 4, forming  $\beta$ -amyloids, probably play a role in channel formation and biofilm dispersion, while RsaE likely regulates the production of  $\alpha$ 2 and  $\alpha$ 3 toxins.

### Keywords:

$\alpha$ PSMs, Biofilm structuring, sRNA-mRNA interaction.

## FAREWELL KEYNOTE

### Invited speaker:

## Tolerance, resistance, and relevance of susceptibility testing?

### Antonio Oliver

*Servicio de Microbiología, Hospital Son Espases, IdISBa, CIBERINFEC. Palma de Mallorca, Spain.*

Some well established biofilm dogmas include (i) Acute infections are caused by planktonically growing bacteria and chronic infections by biofilm growing bacteria (ii) Biofilm growing bacteria show a 100-1000-fold increased antibiotic resistance as compared with planktonically growing bacteria and (iii) Current susceptibility testing methods (either conventional or biofilm specific) fail to predict treatment success in chronic/biofilm-driven infections. While these statements are likely mostly true, a complex interplay of variables related to the susceptibility testing methods, growth conditions, media, microenvironment and parameters analyzed, the pathogen, from individual colonies to whole polymicrobial populations, the infection site, characteristics and persistence drivers, as well as antibiotic pharmacokinetics (PK), clinical breakpoints and treatment outcome variables need to be taken into consideration. Conventional MIC values are likely partially determined by biofilm growth and thus should be informative (to some extent) in biofilm-driven infections, particularly when separating wild-type strains from those producing a “classical” resistance mechanism, proven to play a major role in biofilm growth as well, even if the associated phenotypes sometimes differ from those seen in planktonic growth. A complex repertoire of static and dynamic biofilm susceptibility testing system have been developed, as well multiple pharmacodynamics (PD) parameters (such as MBIC, BBC, BPC, or MBEC) and testing conditions and media, from conventional Müller-Hinton to artificial sputa. Correlations between MICs and biofilm PD parameters are quite low and dependent on the antibiotic and the strain tested. Moreover, current usefulness of biofilm susceptibility testing input appears to be higher for device associated infections than for chronic respiratory infections in CF patients. Natural resistance/tolerance of biofilms does need to be understood and considered, but likely not needed to be proved every day in clinical microlab routine. Conversely, routine biofilm susceptibility testing methods should be as simple as possible (but not simpler) and should permit to differentiate WT from nonWT strains, likely be guided by the definition of biofilm susceptibility ECOFFs. More complex (dynamic) biofilm models, accurately reproducing in vivo conditions and PK/PD parameters, should be used for the design, evaluation and optimization of treatments from a more conceptual perspective. These models need to consider antibiotic resistance dynamics during treatment, which plays a major role in biofilms. Likewise, they need to consider the role of specific acquired resistance mechanisms in biofilms and to understand their associated genotypes and phenotypes. Moreover, understanding the specific differential effects of classical resistance mechanisms in biofilms is also needed. Finally, the very high bacterial population diversity in chronic infections needs to be considered for susceptibility testing and for monitoring treatment outcomes. Thus, whole sample population analysis (and particularly whole population resistome analysis) could be a useful approach to guide treatment strategies and to monitor resistance dynamics in biofilm-driven chronic infections.

## Poster Line Up, Thursday 27 June 13:30-15:00

### BIOFILM EVOLUTION AND REGULATION

PG1-01

#### ToxR as a modulator of surface-associated behaviour in *Pseudomonas aeruginosa*

Dr Manuel Romero<sup>1</sup>, Dr Jean Dubern<sup>2</sup>, Dr Anne Mai-Prochnow<sup>3</sup>, Dr Marco Messina<sup>4</sup>, Dr Eleftheria Trampari<sup>5</sup>, Dr Hardeep Naghra<sup>6</sup>, Prof Kok-Gan Chan<sup>7</sup>, Dr Alessandro Carabelli<sup>8</sup>, Dr Nicolas Barraud<sup>9</sup>, Dr James Lazenby<sup>5</sup>, Dr Ye Chen<sup>2</sup>, Dr Shaun Robertson<sup>2</sup>, Dr Jacob Malone<sup>10</sup>, Prof Paul Williams<sup>2</sup>, Dr Stephan Heeb<sup>2</sup>, Prof Miguel Cámara<sup>2</sup>

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*<sup>9</sup>Genetics of Biofilms Unit, Institut Pasteur, Paris, France, <sup>10</sup>Department of Molecular Microbiology, John Innes Centre, Norwich, UK*

#### Background

*Pseudomonas aeruginosa* can adopt different free-living or biofilm-associated lifestyles. This opportunistic pathogen uses multiple regulators that work together to control the production of virulence factors. These include population-dependent quorum sensing (QS)-mediated and post-transcriptional mechanisms to facilitate adaptation to environmental conditions. In addition, intracellular levels of cyclic diguanylate (c-di-GMP), a global modulator of complex cellular processes at multiple levels: transcriptional, post-transcriptional and post-translational, regulate the lifestyle of this bacterium, with high levels promoting biofilm formation and low levels leading to biofilm detachment and increased motility. In this study, we sought to identify the genetic elements responsible for differences in swarming motility between two sublines of *P. aeruginosa* PAO1: PAO1-Nottingham (PAO1-N) and PAO1-Lausanne (PAO1-L), in order to better understand the interplay between these regulatory mechanisms.

#### Methods

A detailed analysis of the genetic changes between the two sublines, together with functional complementation of swarming motility and biofilm formation, was performed and the identified genes were mutated to study their influence on surface-associated behaviour. Among the identified genetic elements differing between sublines, the association of the ToxR regulator, previously shown to control exotoxin A production, with c-di-GMP regulation was investigated.

#### Results

Genome sequencing revealed a 59kb-deletion containing *toxR* and a mutation in the c-di-GMP-degrading phosphodiesterase BifA in PAO1-N. Among the genes from PAO1-L able to rescue swarming deficiency in a PAO1-N  $\Delta$ smA mutant, *toxR* was found to affect swarming through regulation of the Rhl QS system and subsequent rhamnolipid production. In addition, ToxR was found to tightly bind c-di-GMP and negatively affect traits controlled by this second messenger, including reducing biofilm formation and expression of Psl/Pel exopolysaccharides, necessary for attachment

and matrix scaffolding of sessile communities. Furthermore, a link between RsmA regulation and ToxR expression via the alternative sigma factor PvdS was established.

### **Conclusions**

Here we propose a model for the regulation of surface-associated behaviour by RsmA, BifA and ToxR and demonstrate a previously unknown involvement of ToxR in the c-di-GMP regulated phenotypes of swarming and biofilm formation. This study demonstrates the importance of ToxR in the sophisticated regulation of free-living and biofilm-associated lifestyles, which are suitable for establishing acute or chronic *P.aeruginosa* infections.

### **Keywords:**

Biofilms, Microbial genetics, c-di-GMP

## AHL-based QS signalling promotes uropathogenic E. coli settlement through the de-repression of biofilm formation by SdiA

Celia Mayer<sup>1,2,3,4</sup>, Fadi Soukarieh<sup>3</sup>, Simões Manuel<sup>4</sup>, Jorge Blanco<sup>2</sup>, Miguel Cámara<sup>3</sup>, Dr Manuel Romero<sup>5</sup>

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### Background

Uropathogenic Escherichia coli (UPEC) are among the first pathogens to colonise the urinary tract, being responsible for most of the urinary tract infections and catheter-associated urinary tract infections worldwide. However, other species, such as Pseudomonas aeruginosa, can appear, leading to multi-species infections that can persist by forming microbial aggregates known as biofilms. These highly antimicrobial tolerant complex communities, allows bacteria to establish intra- and inter-specific interactions. Among them, the cell-to-cell signalling communication system or quorum sensing (QS) seems to be involved. Although E. coli does not produce QS autoinducers, it possesses an orphan LuxR-type regulator, SdiA, which can respond to N-acyl-homoserine lactones (AHLs) released by other bacteria. The SdiA-QS system has been described to be involved in the regulation of several virulence-associated phenotypes in E. coli, including biofilm formation. However, the effect of SdiA on UPEC biofilm has not been widely investigated compared to intractintestinal pathogenic E. coli strains. Despite the fact that biofilms are considered a notable public health challenge, the role of the QS on E. coli survival in mixed biofilms has not been studied in depth. Therefore, we used genetic mutation and phenotypic analysis to investigate the influence of the SdiA on biofilm formation by UPEC in the presence or not of exogenous AHLs, in single- and mixed-populations with P. aeruginosa.

### Methods

Single- and dual-species biofilm by UPEC (Wt and  $\Delta$ sdiA mutant) or UPEC co-cultured with P. aeruginosa were grown on coverslips using a Rolling Biofilm Bioreactor (RBB). The effect of AHLs and a LuxR-Ligand addition on biofilms was tested. Confocal scanning laser microscope (CLSM), and CFUs measurements were used to analyse the biofilm formation. To evaluate the specificity of SdiA, bioreporters containing transcriptional fusions with lux operon were used.

### Results

SdiA inhibited biofilm formation in UPEC, and exogenous QS signalling promoted E. coli surface colonisation via SdiA-mediated de-repression of biofilm formation. The low AHL specificity of SdiA was also confirmed.

### Conclusions

This study demonstrates the relevance of SdiA in tightly regulating the free-living and biofilm-associated lifestyles of UPEC.

### Keywords:

Escherichia coli, biofilm, quorum-sensing, SdiA

## Diversity Matters: Complexity as an Evolutionary Pressure on Genotypic Variation in Multispecies Biofilms

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Multispecies biofilms are predicted to be evolutionary hotspots, making them a unique source of information about the impact of diversity on co-evolution and adaptation. Therefore, we conducted a biofilm evolution experiment focusing on two competing strains – *Pseudomonas defluvii* and *Pseudomonas brenneri*, isolated from wastewater facilities – while increasing complexity by adding up to three additional wastewater strains. The experiment lasted for 18 days, during which different combinations (single, dual and multispecies) were analyzed with: CFUs, sequencing, phenotypic screening, and microscopy. *P. defluvii* abundance was low when co-cultured with *P. brenneri*, and further reduced in multispecies combinations. Low abundance in dual- and multispecies biofilms was matched with a drop in phenotypic variation frequency of the dominated species *P. defluvii*. Genes involved in biofilm metabolism and chemotaxis were the most frequently mutated in *P. defluvii*, although mutations were different in variants isolated from mono-, dual- and multispecies biofilms. These results strongly suggest that the complexity in the biofilm community impacts genotypic diversification over extended periods of time (18 days). Oppositely, phenotypic variation of the dominating species, *P. brenneri*, increased with ascending diversity, albeit to a lesser extent. Several evolved *P. defluvii* variants were selected based on phenotypic changes (wrinkled colonies) compared to wild-type colonies (smooth colony surface). These variants showed increased performance when grown with *P. brenneri* in a different 10-day evolution experiment. This demonstrates the impact of diversity on evolution in complex biofilms by proving that interspecific genotypic variation found in biofilm formation and interspecies interactions compensates for intraspecific changes over extended periods of time. Moreover, co-evolution patterns are still hard to predict, as shown by the different behaviors in *P. defluvii* and *P. brenneri*, highlighting the relevance of this type of research. Our multispecies biofilm long-term evolution experiment a in this study represents a significant step forward towards a better understanding of the complex dynamics of biofilms in nature, and has shown that co-existence with three to five species drives adaptation of two focal species similarly to biofilm lifestyle, suggesting that diversity defines adaptation through genotypic adaptation.

### **Keywords:**

multispecies biofilm, genotypic adaptation, co-evolution

## A mutation in *icaR* leads to a mucoid phenotype of *Staphylococcus argenteus* in an individual with cystic fibrosis

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### Background/Aims

*Staphylococcus argenteus* is a newly identified species of coagulase-positive staphylococci, exhibiting a high phenotypic similarity and close genetic relation to *Staphylococcus aureus*. Unlike *S. aureus*, *S. argenteus* has rarely been reported to play a role in chronic or recurrent infections, including pulmonary disease in people with cystic fibrosis (pwCF). In a recent multicenter study, in which we determined the prevalence of mucoid *S. aureus* in the airways of pwCF, we discovered for a single participant *S. argenteus* besides *S. aureus*. Of note, shortly after the detection of non-mucoid penicillin-susceptible (PS) *S. argenteus*, a mucoid PS variant occurred, which was then later replaced by *S. aureus* when the patient was treated with cefuroxime.

### Methods

Throat swabs from five consecutive quarterly visits of this particular pwCF were sent to our microbiological laboratory. Ten isolates were randomly selected from each specimen of each visit for detailed analysis. Identification of isolates was conducted using MALDI-TOF MS, *spa*-typing and whole-genome sequencing. Phenotypic characterization of isolates with respect to biofilm formation/ mucoidy was assessed using a static microtiter plate assay. In vitro competition assays were carried out to mimic the in vivo situation.

### Results

Isolates within each species belonged to the same *spa* type. In case of *S. argenteus*, mucoidy was caused by a mutation in the repressor of the intercellular adhesion (*ica*) operon, leading to unimpeded polysaccharide-dependent biofilm formation. In vitro growth analysis demonstrated that *S. argenteus* grew faster and thus replaced *S. aureus*, with the mucoid *S. argenteus* dominating. Surprisingly, under sub inhibitory concentrations of cefuroxime the situation was exactly the opposite.

### Conclusion

In this study, we described for the first time the isolation of mucoid *S. argenteus* co-infecting the airways of a person with CF together with *S. aureus*. Interestingly, the mucoid *S. argenteus* strain carried a mutation in *icaR*. Further research should try to define the role of *S. argenteus* as a potential pathogen in CF.

### Keywords:

CF, *S. argenteus*, biofilm, *ica* operon

## Aspergillus fumigatus biofilm: Role of chemically diverse and multifunctional molecules

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### Background

During infection and colonization, *A. fumigatus* grows as a colony characterized by multicellular and multilayer hyphae embedded in a matrix. This type of growth is compatible with the definition of biofilm. Little is known about the differential characteristics of these structures among strains and their role in their infective capacity. Aims: To describe the *A. fumigatus* biofilm development adopting a dynamic biological approach, through the qualitative and quantitative analysis of diverse molecules, generated as a consequence of cell-cell interaction, using phenotypically different strains implied in diverse pathologies.

### Methods

Biofilm structure was developed in RPMI 1640 medium with 2% glucose, 2 inoculum size (10<sup>4</sup>-10<sup>6</sup> cfu/ml) and in a microplate format. Seven different strains of *A. fumigatus* were used: 1 reference strain, 2 clinical isolates from patients with Invasive Aspergillosis (IA), 2 clinical isolates from patients with Cystic Fibrosis (CF) and 2 environmental isolates (1 susceptible, 1 resistant strains). Mature biofilms were developed at 37°C and different chemical analysis were performed. For biofilm characterization, biomass was assessed by crystal violet staining and cell viability was tested via XTT incubation. Extracellular matrix (ECM) was also extracted and characterized. Capacity to activate secondary metabolism was also evaluated by quantification of the secondary metabolite gliotoxin (GT), by using a chromatographic method.

### Results

All strains developed the biofilm structure. Significant differences were observed among the strains, mainly in terms of cell viability and GT production. Clinical strains from CF patients showed higher cell viability and lower GT production. We found the opposite pattern (lower viability and higher GT production) in isolates from patients with IA. Differences in terms of biomass (CV) or ECM weight were insignificant between most of the strains studied.

### Conclusions

1) The in vitro *A. fumigatus* biofilm model developed allows the differential characterization of this common growth mode in nature. 2) Secondary metabolism activation occurs during the biofilm maturation, and determines the release of GT. Production of GT follows a strain- and time-dependent pattern. 3) Strains origin seem to influence characteristics of the biofilm generated, showing structural, metabolic and also dynamic differences in secondary metabolism activation between strains with colonizing/ invasive profiles.

### Keywords:

dynamic, extracellular matrix, secondary metabolism

## Unlocking cryptic expression of secondary metabolites and surface-associated polysaccharides in *Burkholderia pseudomallei*.

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### **Background**

*Burkholderia pseudomallei* (Bp) is the causative agent of melioidosis, a severe infectious disease that poses a significant threat globally. Given its widespread distribution, high mortality rates, antibiotic resistance, and environmental persistence, it is crucial to understand how it responds to environmental signals to adapt, especially in the context of biofilm production and host infection. Bp exhibits robust biofilm formation, enabling it to adhere to surfaces and establish antibacterial tolerant communities. Surface-associated polysaccharides contribute substantially to biofilm growth and protection during infection. Bp produces a diverse array of surface-associated polysaccharides, which contribute to the robustness and adaptability of Bp. Additionally, it produces an array of secondary metabolites during biofilm growth. Our aim is to understand the specific role of these molecules in biofilm development and infection.

### **Methods**

To better understand the regulation of these molecules we used a variety of approaches that relied on disrupting c-di-GMP signaling in Bp and altering the environmental conditions. A combination of bioinformatics analyses and untargeted metabolomics were used to characterize alterations in the production of secondary metabolites and surface-associated polysaccharides under varying growth conditions. This was done in parallel with studies that integrate transcriptional profiling with genetic deletions targeting c-di-GMP regulatory components.

### **Results**

Our research demonstrates that disruption of c-di-GMP messaging through mutational analyses and conditional expression studies of c-di-GMP metabolic genes reveal phenotypic impacts on biofilm formation, colony morphology, motility, and secondary metabolite expression in response to environmental cues. Differential expression of surface-associated polysaccharides was also observed during cellular and host infection.

### **Conclusions**

The ability to produce numerous surface-associated polysaccharides and secondary metabolites in Bp is context-dependent. Our research approach has the potential to find the keys to understand the cryptic expression and regulation of key components that contribute to biofilm growth dynamics and infection. The genetic backgrounds and environmental growth conditions identified in these studies will allow further biochemical characterization of these cryptic molecules and their role in growth, survival, competition, and infection.

### **Keywords:**

c-di-GMP, exopolysaccharides, The Great Adapter

## The effect of Antioxidants-Ciprofloxacin combinations on *Pseudomonas aeruginosa* Biofilms

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### Background

Development of antimicrobial resistance (AMR) has become one of the severe health threats in the current century. Biofilm infections represent therapeutic challenges requiring prolonged antibiotic treatment due to the intrinsic tolerance to antimicrobials and immune system of biofilm-growing bacteria. We have previously shown, that formation of reactive oxygen species (ROS) contributes to the bactericidal effect of ciprofloxacin (CIP) on *P. aeruginosa* biofilms. The mutagenic potential of ROS is of concern for the development of mutational AMR, pointing out the importance of investigating the impact of antioxidants on mitigating AMR.

### Materials & Methods

We explored AMR evolution in CIP-treated *P. aeruginosa* biofilms using both dynamic flow cells, and static glass beads models through three passages. The strain PAO1-mCherry-PCD-gfp monitoring mutations in *nfxB*, the negative regulator of the MexCD-OprD efflux pump, was used. Three different antioxidants (Edaravone, N-Acetyl-L-cysteine, and Thiourea) were tested for their impact on reducing AMR when combined with CIP, individually. Three-day-old flow-cell biofilms were exposed to CIP treatment, CIP + antioxidant, or controls, and images were captured on three consecutive days. The harvested population from flow cells was the whole genome sequenced WGS.

### Results

The confocal laser microscopy images of flow cells system showed a general trend of a significantly higher rate of *nfxB* (gfp) mutations in the CIP-treated cells compared to the CIP + antioxidant treatment for all the three used antioxidants, which was also confirmed by WGS. However, the population analysis of biofilm populations from both the glass beads and flow cells biofilms showed that the antioxidants Edaravone and N-Acetyl-L-cysteine didn't significantly impact the evolution of CIP resistance. In contrast, thiourea a scavenger of hydroxyl radicals, reduced significantly the size of the resistant population that grew on 0.5 mg/L CIP compared to the CIP-treated biofilms in the last two passages ( $p = 0.001403$  and  $p = 0.004324$ , respectively). The MICs of isolates collected after CIP+ thiourea treatment (0.1 – 0.75 mg/L) were lower compared to CIP-treated cells, which reached 3 mg/L MIC.

### Conclusions

These results underscore the context-dependent effects of antioxidants on AMR dynamics, providing valuable insights into potential strategies for mitigating resistance in bacterial populations.

### Keywords:

AMR, Evolution, antioxidants

## Deciphering the cyclic-di-GMP signalling network in *Pseudomonas aeruginosa*

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*Pseudomonas aeruginosa* causes hospital-acquired chronic infections in immunocompromised patients and also due to its ability to form biofilms on medical devices. Cyclic di-guanosine monophosphate (c-di-GMP) is an important intracellular secondary messenger involved in regulating biofilm formation with 41 diguanylate cyclases (DGCs with GGDEF domain) and phosphodiesterases (PDEs with EAL or HD-GYP domains) that make and break c-di-GMP, respectively, in *P. aeruginosa*. It is currently unknown how these many DGCs and PDEs use a single diffusible secondary messenger, c-di-GMP, to operate distinct pathways with specific outputs. Our aim is to decipher the interconnections and pathways of specific DGCs and PDEs to reconstruct the c-di-GMP network in *P. aeruginosa*. One approach was deleting individual 41 DGC/PDE genes in *P. aeruginosa* PAO1 and assessing each mutant in terms of biofilm, virulence, and motility phenotypes. Cumulative deletion of all 41 DGC/PDE genes in PAO1 is on-going to create a single strain depleted of c-di-GMP-related enzymes. This is a powerful tool that will be used to investigate the contribution of each individual DGC/PDE. Another approach is to characterise specific enzymes and understand their role in regulating c-di-GMP and related phenotypes with an example being a dual domain, PA0285, which contains both a GGDEF and an EAL domain. Downstream effectors and interacting partners will be identified through RNA-seq and protein pulldown assays. Cumulative deletion of 15 PDEs in a single strain showed a 5-fold-increase in biofilm attachment compared to PAO1. Characterisation of PA0285 show that although it is a dual domain enzyme, it exhibits potent PDE activity but no DGC activity in vitro. Deletion of this gene led to significant increased attachment at early stage of biofilm development. RNA-seq analysis further revealed that PA0285 may be involved in regulation of fimbrial genes. Ongoing work will focus on elucidating the role of DGC/PDEs and the downstream effectors. Assessment of the different DGC/PDEs is crucial to uncover the complexity of the c-di-GMP network from its regulation to effector binding and eventual phenotypic output in *P. aeruginosa*. Controlling biofilm formation and dispersal by manipulating c-di-GMP makers and breakers will help provide solutions to eliminate hard-to-treat biofilm in chronic infections.

### Keywords:

*P. aeruginosa*, c-di-GMP, biofilm regulation

## Bacillus thuringiensis' Evolutionary Dance in Mixed-Species Communities: Insights from Experimental Evolution

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### Background

Bacterial biofilms represent intricate ecosystems where dynamic interactions between microbial communities and their environment play a pivotal role in shaping evolutionary trajectories. *Bacillus thuringiensis*, renowned for its adaptability and applications, offers a valuable model for studying how environmental factors, abiotic and biotic, shape biofilm evolution.

### Aim

This study elucidates how varying environmental conditions, including nutrient availability, lifestyle, and interspecies interactions, modulate the adaptive responses and genetic diversity within *B. thuringiensis* biofilms.

### Methods

Combining experimental evolution, genome sequencing, and proteomic analysis, we examined the impact of individual environmental parameters on genetic diversification. We also characterized glycan and protein matrix components using fluorescent lectin staining and matrix proteomics in mono- and mixed-cultures.

### Results

We observed the consistent emergence of a phenotypic variant in *B. thuringiensis* biofilms, regardless of the conditions. This variant exhibited distinct phenotypic traits, including reduced Congo red binding and sporulation deficiency. Genomic analysis revealed mutations in *spo0A*, a key sporulation regulator while analysis of growth parameters in planktonic cultures showed a significantly shorter generation time than its ancestor. Selection of this variant was significantly favored in biofilm settings and when coexisting with other species co-isolated from a wastewater facility, namely *Pseudomonas defluvii* and *Pseudomonas brenneri*. Strikingly, the dominance of the phenotypic variant within biofilms did not translate into improved biofilm formation compared to its ancestor. In fact, biofilm matrix production was reduced in mixed-species biofilms, either through self-production or induction in *Pseudomonas* species. Matrix proteomics confirmed these findings, showing a decreased abundance of *TasA*, a major biofilm matrix component in *Bacillus* species, and *SpoVG*, a regulator of sporulation and biofilm formation. This reduction aligns with the observed sporulation deficiency in the variant.

### Discussion

Despite its growth advantage, the evolved variants coexisted with the ancestor or *Pseudomonas* species, suggesting niche partitioning within the biofilm. The variant may dominate certain regions while the ancestor could still persist in others. From an application perspective, these variants may exhibit enhanced metabolic activity, growth rates, or tolerance to environmental stressors, making

them well-suited for use in specific bioprocessing applications, such as biocatalysis or bioproduction of valuable compounds.

**Keywords:**

biofilms, adaptive evolution, microbial interactions,

## Understanding Genomic Adaptations of *Pseudomonas aeruginosa* in Chronic Wounds

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### Background

*Pseudomonas aeruginosa*, a gram-negative opportunistic pathogen, is renowned for its biofilm-forming capabilities and prevalence in chronic infections like cystic fibrosis (CF) and chronic wounds. Its expansive genome enables adaptation to diverse environments, mainly explored in CF. This study aims to delve into clonal diversity and genomic adaptations of *P. aeruginosa* in the unique context of chronic wounds.

### Methods

We collected longitudinal isolates (n=123) of *P. aeruginosa* from the chronic wounds of 4 patients in a non-interventional cohort. We conducted whole-genome sequencing to characterize genetic features and phenotypic assays to evaluate antimicrobial resistance (AMR), motility, virulence and biofilm formation functions.

### Results

Isolates from the same patient exhibited identical multi-locus sequence types (MLSTs) (132, 303, 446, 641). Nevertheless, we observed AMR and virulence gene pattern variability within the same patient. Phenotypically, 28% and 16% of isolates tested negative for protease production and twitching motility, respectively. We found differing tolerance levels to the antimicrobials ciprofloxacin and piperacillin/tazobactam within the same MLSTs. Ongoing analysis aims to correlate differences in gene content and genetic variants with observed phenotypes.

### Conclusions

While MLSTs suggest the dominance of a single *P. aeruginosa* clone upon wound entry, the variability in resistomes and virulomes indicates adaptation through horizontal gene transfer within each clonal lineage. Over the following months, we will study whether adaptation extends to the loss/gain of core genes and patho-adaptive mutations, as found in CF studies. We hope to shed light on the driving forces of evolution in chronic infections in general, and chronic wounds in particular.

### Keywords:

*Pseudomonas aeruginosa*, chronic wounds, adaptation

## AlgR is an activator of *cdrA* expression in *Pseudomonas aeruginosa*

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### Background

Regulation of biofilm formation and the synthesis of extracellular matrix structural components in *Pseudomonas aeruginosa* is complex. New layers of complexity are continuously elucidated, and this knowledge is an important aid in the search for potential anti-biofilm drug targets. Targets that can be exploited in the pursuit of new treatment regimens against this tenacious opportunistic pathogen involved in many chronic and nosocomial biofilm-based infections. The aim of this study was to elaborate on the biofilm-specific regulatory role of the AlgR transcriptional regulator beyond its well-described role in regulating alginate biosynthesis.

### Methods

A defined knockout of *algR* was created in various mutants derived from *P. aeruginosa* PAO1. Additionally, site-directed mutagenesis of the canonical AlgR binding site located in the *cdrA-hprA* intergenic region was performed both chromosomally, and on the cyclic di-GMP responsive monitor plasmid pCdrA-gfp. Different assays, e.g. phenotypic and bioreporter-based, were used to describe the influence of AlgR on *cdrA* expression and regulation of CdrA-related biofilm traits in *P. aeruginosa* PAO1.

### Results

Absence of AlgR significantly decreased the fluorescent output from the pCdrA-gfp bioreporter. Investigation of the potential mechanism revealed a canonical AlgR binding site in the *cdrA-hprA* intergenic region. Although located on the complementary strand relative to the *cdrA* promoter, site-directed mutagenesis of the binding site on the *cdrA-gfp* monitor led to a decrease in the fluorescent output similar to that observed when AlgR was absent. This indicated that AlgR most likely functions as a transcriptional activator of *cdrA* expression through direct binding to the *cdrA* promoter region. Hyperaggregation in liquid culture and colony morphology assays confirmed the involvement of AlgR in positively regulating phenotypes involving CdrA.

### Conclusion

The finding adds a new level of complexity to the regulatory role of AlgR in *P. aeruginosa*. Expression of *cdrA* is positively regulated by AlgR in addition to the positive regulation by the second messenger cyclic di-GMP. AlgR is thus a regulator of both alginate biosynthesis and the CdrA adhesin in *P. aeruginosa*.

### Keywords:

*Pseudomonas*, biofilm, adhesin, transcriptional regulation

## Investigation of the role of oxidative stress in the evolution of *Pseudomonas aeruginosa* biofilms using a mutagenesis reporter

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### Background

Commonly used antibiotics on *P. aeruginosa* biofilms are thought to elicit the production of reactive oxygen species (ROS) as part of their bactericidal effect. Antioxidant mechanisms hence play an important role in bacterial survival during antibiotic treatment. The absence of the major catalase, KatA, in *P. aeruginosa* may contribute to increased mutagenesis and faster development of resistance when cells are exposed to subinhibitory concentrations of antibiotics. The aim of this study is to establish a fluorescent transcriptional reporter for *nfxB* mutant detection in  $\Delta katA$  *P. aeruginosa* background to investigate the role of oxidative stress in mutagenesis within biofilms. The *nfxB* mutants are overexpressing the MexCD-OprJ multidrug efflux pump.

### Methods

An in-frame deletion of the gene encoding the catalase KatA (*katA*) was introduced into the PAO1-mCherry-PmexCD-gfp+ monitor strain through allelic exchange. A deletion allele was cloned into an allelic exchange vector and then inserted into the bacterial chromosome by conjugation. A flow-cell system was used to study biofilm evolution of the wild-type and  $\Delta katA$  monitor strain. The occurrence of *nfxB* mutations was followed by confocal laser scanner microscopy and the acquired images were processed with Imaris.

### Results

Successful deletion of the *katA* gene encoding KatA, an enzyme responsible for scavenging ROS, was confirmed by a negative result in the catalase test. The absence of further mutations was confirmed by sequencing.

The wild-type and  $\Delta katA$  version of the PAO1-mCherry-PmexCD-gfp+ monitor strain were cultivated in a flow-cell system. After 96 h of growth, biofilms were either treated with subinhibitory concentrations of ciprofloxacin for 24 h or used as controls. In both cases, the microcolonies showed constitutive red fluorescence and smaller areas with green fluorescence indicating mutations in *nfxB*. Preliminary analysis of the confocal images after ciprofloxacin treatment suggests a larger subpopulation with *nfxB* mutations in the  $\Delta katA$  mutant compared to the wild type.

### Conclusions

The production of mutagenic ROS upon ciprofloxacin treatment results in an increased resistant subpopulation due to *nfxB* mutations in *P. aeruginosa* cells lacking the KatA catalase.

### Keywords:

*Pseudomonas aeruginosa*, flow-cell, oxidative stress

## Biocides drive rifampicin cross-resistance in Escherichia coli biofilms

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### Background

The understanding of phenomenon promoting antibiotic resistance from farm to fork is a world health priority. Evidences suggested that the massive use of disinfectant biocides in food industries may constitute a contributing selective factor. However, bacterial survival strategies to biocides and cross-resistance development remain unclear, particularly the role of 3D-structured biofilm lifestyle, which can greatly influence stress adaptation. In this study, we investigated biofilm adaptation to biocides commonly used on the food chain and its effects on antibiotic resistance emergence in *E. coli*.

### Methods

Biofilms of 8 *E. coli* strains isolated along the food chain were exposed during one month to 4 biocidal substances: benzalkonium chloride, polyhexamethylene biguanide, triamine (N- (3-aminopropyl)-N-dodecylpropane-1,3-diamine) and sodium hypochlorite. Emergence of resistance to ciprofloxacin, rifampicin and gentamicin were then monitored by plating biofilm bacteria every week on selective media. Resistant variants were quantified and characterized through phenotypic and genomic analyses (WGS).

### Results

Exposure to triamine and benzalkonium chloride increased significantly the quantity of rifampicin-resistant clones collected when comparing to controls exposed to water. WGS of 154 clones revealed recurrent genetic targets associated with each biocide adaptation. A strong association was especially observed between triamine-selected variants and mutations in the lipopolysaccharide biosynthesis genes, especially genes involved in O-antigen biosynthesis. Interestingly, while these variants mostly exhibited low-level resistances to rifampicin, the presence of mutations did not affect their fitness, and even increased growth abilities in suspension in presence of biocides for several variants compared to parental strains. Moreover, most variants overproduced matrix components and outcompeted their parental strains in mixed-biofilms as revealed by confocal laser scanning microscope observations. Overall, results suggested that LPS-associated mutations led to a bacterial adaptation to biocide both at the cellular and collective (biofilm) levels.

### Conclusions

This work supports a possible effect of biocides on selection of antimicrobial resistance in bacterial communities. The identification of the LPS biosynthesis pathway as a key target involved in adaptation to triamine and antibiotic cross-resistance demonstrates the existence of recurrent mechanisms of adaptation. Such targets could be harnessed as markers for cross-resistance surveillance to better monitor the impact of biocide usage.

### Keywords:

Biofilms, Biocides, AMR Resistance, Rifampicin

## Cyclic di-GMP controls biofilm formation and key virulence factors in multidrug resistant *Acinetobacter baumannii*

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### Background

Carbapenem-resistant *Acinetobacter baumannii* is listed by the World Health Organisation as a critical priority pathogen, for which novel therapeutics are urgently needed. *A. baumannii* is able to form strong biofilms that help it tolerate antimicrobial treatment. Despite the relevance of this pathogen, little is known about how it regulates biofilm formation. Here, we aim to elucidate how the second messenger cyclic di-GMP (cdGMP) controls biofilm formation in this species.

Methods: We used the multidrug resistant (MDR) clinical isolate *A. baumannii* AB5075 and applied genetic tools to control its cdGMP levels. After validating the changes in cdGMP amounts using a fluorescent biosensor, we performed a differential RNA-seq (dRNA-seq) to uncover the cdGMP-driven global transcription changes and its targets. The regulation of specific gene groups was validated at the phenotypic level by biofilm formation and Congo red assays, as well as motility experiments. Furthermore, we performed conjugation assays and used the *Galleria mellonella* virulence model to assess how cdGMP levels affect these behaviours.

### Results

To control the cdGMP levels in AB5075, we inserted the heterologous diguanylate cyclase *pleD\** or the phosphodiesterase *rocR* under an inducible promoter in a neutral site in the chromosome. We validated the changes in cdGMP levels after the induction of these genes by using an MDR *Acinetobacter*-adapted fluorescent biosensor. After this, we performed a dRNA-seq experiment comparing cells expressing either *pleD\** or *rocR* (high versus low cdGMP). This revealed that cdGMP directly upregulates genes involved in surface attachment and production of exopolysaccharide matrix. Additionally, cdGMP produced the downregulation of the genes implicated in twitching motility. These behaviours were tested experimentally, validating our transcriptomics results. Strikingly, we observed an increased conjugal transfer of native AB5075 plasmids correlating with cdGMP-induced high biofilm formation, therefore promoting the acquisition of plasmid-borne antimicrobial resistance genes. Lastly, we established that high cdGMP levels correlate with a lower virulence of AB5075 in vivo in *G. mellonella*.

### Conclusions

Our results demonstrate that cdGMP signalling controls biofilm formation in the critical-priority pathogen *A. baumannii* to the detriment of motility. Furthermore, this higher biofilm formation correlates with an increased plasmid transfer and a reduced virulence in vivo.

### Keywords:

Regulation, Second messengers, Virulence, Conjugation

## Can we control biofilm-induced clogging in porous media?

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In bioengineering systems using biofilms in porous media to produce proteins of interest or to treat wastewater, a key limiting factor is fouling leading to clogging, which may cause costly damages and production losses. The large pressure drops variations coming from the competition between growth and detachment is not sustainable for a continuous process. In this work, we ask the question of whether we can avoid such fluctuations and more generally control the permeability of porous structures colonized by biofilms. Our ultimate goal is to fabricate a proof-of-concept system allowing us to reach a target permeability in a microfluidic device through a close loop control.

We grow a model biofilm of *Pseudomonas aeruginosa* in a microbioreactor technology that allows us to measure the dynamics of the pressure drop across a porous structure. The porous structure is 3D printed with high-resolution stereolithography with channels 300µm wide over 700µm long, a connectivity of 3 and total porosity of 9%. With this system, we can dynamically regulate environmental parameters – porous structure, nutrient types, temperature with a thermostat cell and flow rate through programmable gear pumps– while also measuring the effect of those different parameters through a pressure sensor and map the reactivity of the biofilm.

Our preliminary results on biofilm's growth in porous media show a pressure drop that follows 3 phases: an initial phase with attachment and microcolony growth, a phase with a fast increase in the pressure drop with a thickening of the biofilm until 150 mbar, and finally a dynamic equilibrium phase where the competition between growth and detachment leads to large amplitude fluctuations of the pressure drop. In particular, changes in the temperature and nutrient type affect these dynamics, especially oscillatory frequencies and amplitudes. This allows us to get the building blocks to develop a transfer function for feedback control of the permeability.

This enables us to tune our inputs to control biofilm-induced pressure drop and discuss the implications in terms of the definition of a cost function. Besides acting on physicochemical conditions, biological actuator could be implemented with the addition of a predator as *Bdellovibrio bacteriovorus*.

### **Keywords:**

3D-printed porous media, permeability, control

# INNATE AND ADAPTIVE IMMUNE RESPONSES TOWARDS BIOFILM INFECTIONS

PG1-16

## Staphylococcus aureus SaeR/S regulated factors overcome human complement mediated inhibition of aggregation to evade neutrophil killing

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### Background

*Staphylococcus aureus* is a frequent culprit in implant-associated infections and employs an impressive collection of virulence factors to escape killing by the host immune system. The specific immune evasion strategies *S. aureus* employs during early biofilm formation on a surface are still relatively unknown. The overall objective of this study was to investigate the molecular basis of this effect.

### Methods

Time-lapse confocal microscopy was employed to quantify interactions between *S. aureus* biofilm aggregates and human neutrophils in vitro. Bacterial isogenic deletion mutants and sera affected in various complement proteins were used to identify specific mechanisms of tolerance to neutrophil killing.

### Results

Surface associated *S. aureus* rapidly formed small biofilm aggregates when grown in human serum. Aggregates began to show individual and population level tolerance to neutrophil killing upon reaching a size of approximately 50  $\mu\text{m}^2$ . The SaeR/S two-component gene regulatory system was essential for the formation of cohesive aggregates and subsequent neutrophil evasion. Inhibition of LAC $\Delta$ saePQRS aggregation required serum complement, as complement inhibition restored biofilm aggregation. The ability to prevent LAC $\Delta$ saePQRS aggregation was dependent on complement proteins C3 and factor B, but not complement C4 or C5, suggesting that this activity occurs at the level of the alternative C3 convertase, C3bBb or the alternative C5 convertase, C3bBbC3b. Several redundant complement inhibiting genes regulated by SaeR/S specifically target the alternative convertases and combined to facilitate aggregation of wild type bacteria. Deletion of these seven genes ( $\Delta$ C7) resulted in impaired aggregation in human serum.

### Conclusions

These data demonstrate a novel function of serum complement - the ability to inhibit bacterial aggregation. *S. aureus* potently inhibits this effect through the production of multiple complement interfering proteins that are regulated by the SaeR/S system. These findings provide key insights into the pathogenesis of *S. aureus* biofilm infections.

### Keywords:

biofilm, Staphylococcus, complement, neutrophil, aggregation

## Immunomodulatory effects of oral streptococci

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### **Background incl. aims**

The oral microbiome is a critical component of oral health and disease. Bacteria exist in biofilms, in close communication with one another and the human host. Within these communities *Streptococcus* spp. play an important role in health (eubiosis) but also in response to changes in environmental conditions that can lead to oral disease (dysbiosis). Periodontitis is a significant oral inflammatory disease of the supporting tissue of the teeth. The local inflammatory response is coordinated by intercommunicating immune cells and mediators that respond to bacterial stimuli leading to an overstimulation of the immune response and tissue damage. Platelets recognize bacteria and participate in inflammation by release of mediators, binding to neutrophils and monocytes, modifying their immune function. We propose that platelets may contribute to the pathogenesis of periodontitis. We are therefore investigating the effect of different *Streptococcus* spp. associated with health or disease on innate immune cell activation in blood: platelets, neutrophils, and monocytes.

### **Methods**

Flow cytometry and ELISA is used to assess platelet, neutrophil and monocyte activation in whole blood after incubation with washed populations of *S. gordonii*, *S. parasanguinis*, *S. oralis* and *S. constellatus*. Neutrophil activation is assessed by upregulation of surface CD11b, CD66b and release of myeloperoxidase. Monocyte activation is assessed by upregulation of CD11b, HLA-DR and cytokine production. Platelet activation is determined by upregulation of surface CD62P, CD63 and the formation of Platelet-Leucocyte complexes.

### **Results**

Our preliminary results have demonstrated bacteria-specific profiles of innate immune cell activation. In particular, distinct patterns of activation have been observed for neutrophils and monocytes exposed to *Streptococcus gordonii* as compared to *Streptococcus constellatus*.  
Conclusions: Our findings highlight the need to investigate the immunomodulatory effects of distinct species of *Streptococcus* associated with oral health and disease.

### **Keywords:**

streptococci, platelets, innate immunity

## Biofilm-dispersed pneumococci induce elevated leukocyte activation and platelet activation

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### Background incl. aims

*Streptococcus pneumoniae* (the pneumococcus) effectively colonizes the human nasopharynx. However, the bacteria can migrate to other host sites and cause infections such as otitis media, pneumonia, and sepsis, resulting in 1-2 million deaths annually. Previous pneumococcal studies indicate that biofilm-grown bacteria and bacteria released from biofilms in response to “danger signals” (i.e., dispersed bacteria) represent populations associated with phenotypes of bacteria during colonization and transition to disease, respectively. How these niche-adapted populations interact with immune cells upon reaching the vascular compartment has not previously been studied. Here, we investigated neutrophil, monocyte, and platelet activation using ex vivo stimulation of whole blood and platelet-rich plasma with pneumococcal populations associated with distinct stages of the infectious process (biofilm and dispersed bacteria) as well as conventional broth-grown culture (planktonic bacteria).

### Methods

Flow cytometry and ELISA were used. Neutrophil and monocyte activation were assessed by upregulation of surface CD11b and plasma myeloperoxidase. Platelet-neutrophil complex formation was assessed as a surrogate marker for platelet activation. Platelet activation was determined by degranulation of alpha and dense granules. Alpha granules were assessed by upregulation of surface CD62P and plasma CD62P and PF4. Dense granules were assessed by upregulation of surface CD63 as well as mepacrine release/uptake. Platelet functionality post-bacterial stimulation was assessed by subsequent response to thrombin receptor activator peptide 6 (TRAP-6). Lastly, we examined bacteria association to the immune cells.

### Results

Overall, biofilm-derived (biofilm bacteria and dispersed bacteria) induced activation of neutrophils, monocytes, and platelets. In contrast, little to no activation was induced by planktonic bacteria. Platelets remained functional after stimulation to bacterial populations and responsiveness to TRAP-6 was inversely related to initial activation. Bacteria association with the immune cells followed a similar pattern as activation.

### Conclusions

This study highlights bacterial population differences in activation of and association with immune cells, emphasizing the importance of considering bacterial growth conditions, especially for pathogens that have a biofilm (and triggered dispersal) state. A focus on distinct bacterial populations associated with stages of disease development will move forward the understanding of host-pathogen interactions and may help identify novel immunomodulatory targets to interfere with the infectious process.

### Keywords:

activation monocytes neutrophils platelets pneumococci

## Comparative Analysis of Gene Expression and Cytokine production driven by *Candida albicans* Biofilms and Planktonic Cultures

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### **Background and Aim**

*Candida albicans* is a ubiquitous fungus known for its polymorphic growth as yeast and hyphae, and its ability to grow planktonically or as biofilms. Given the involvement of this species in human infections, it is important to understand its effect on host immunity. This study compared the expression of genes involved in host immunity and cytokine production by THP-1-derived macrophages following exposure to heat-killed *Candida albicans*. The aim was to explore the dynamics of host cell-pathogen interactions and identify potential therapeutic targets.

**Methodology:** *Candida albicans* SC5341 was cultured under conditions that promoted its different morphologies (yeast and hyphae). The yeast form was grown in Sabouraud dextrose broth, while the hyphal form was generated in RPMI 1640 with 10% foetal bovine serum. Biofilms were developed in static batch culture and CDC bioreactors for yeast and hyphal forms, respectively. Post-culture, specimens were heat-inactivated and exposed to THP-1-derived macrophages. RNA extraction, followed by Nanostring nCounter technology was used to screen cytokine gene expression. TNF- $\alpha$  and IL-10 levels were confirmed by ELISA.

### **Results**

NanoString nCounter analysis indicated significant alteration of macrophage gene expression (e.g. upregulation of EBI3, IL1B, CCL8, and IL8) after exposure to heat-killed hyphae and yeast, in both planktonic and biofilm forms. Biofilm growth led to greater upregulation of genes (EBI3, IL1B, CCL8, and IL8) compared with planktonic growth. ELISA showed elevated production of TNF- $\alpha$  following hyphal biofilm challenge and an increase in IL-10 expression for yeast forms and biofilms, although the latter was after a longer period of exposure time to *C. albicans*.

### **Conclusion**

The study highlighted distinct immune responses to *C. albicans* growth, with heightened immune responses to hyphal forms and biofilms. Future work will include RT-qPCR validation of key upregulated genes (EBI3, IL1B, CCL8, and IL8) identified by Nanostring analysis, which will further confirm the immune response mechanisms against these different types of *C. albicans* growth.

### **Keywords:**

Bioiflm. Immunity. *Candida albicans*. Planktonic

## LESS KNOWN BIOFILM INTERACTIONS

PG1-20

### Investigating the Impact of Indigenous Multispecies Biofilm Formation on the Bioaugmentation Process for the Removal of Emerging Contaminants

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#### Background

The bioaugmentation, the injection of microorganisms capable of degrading contaminants into a targeted area, holds the potential for enhancing the sustainable removal of emerging contaminants. The application of bioaugmentation to biological filtration systems (BFS) for drinking water production has received much attention for various emerging contaminants including harmful cyanotoxins. Successful bioaugmentation primarily relies on the survival and activity of bioaugmented bacteria and their interactions with endogenous multispecies biofilms (MSB) in BFS. However, the influence of endogenous MSB on bioaugmentation and biodegradation processes remains poorly understood. This study aimed to assess the impact of MSB on the bioaugmentation of a cyanotoxin-degrading bacterium, their interspecies interactions, and biodegradation of a cyanotoxin.

#### Methods

Biodegradation experiments were conducted using lab-scale column reactors inoculated with MSB obtained from a local water treatment plant. Bioaugmentation involved introducing a cyanotoxin degrader into these column reactors after confluent MSB development. Biofilm formation and the activity of the bioaugmented cyanotoxin degrader were monitored through adenosine triphosphate analyses and quantitative polymerase chain reaction, respectively. Microbial community structure analyses were also performed to trace the changes in the bacterial community before and after the bioaugmentation. Further, water quality and fluorescence excitation emission matrix analyses were conducted to assess the performance of BFS for the removal of natural organic matter in water.

#### Results

Obtained results demonstrated that MSB contributed to increased biofilm formation and activity of the cyanotoxin-degrader, enhancing cyanotoxin removal. The complete removal of the target cyanotoxin as observed within 4 days after bioaugmentation, suggesting a synergistic role of MSB on the bioaugmentation process. The degradation activity of cyanotoxin-degrading bacteria, as indicated by mlrA gene (a bacterial gene associated with cyanotoxin biodegradation) expression, was 3 times higher in the bioaugmented columns. Additionally, 16s rRNA sequencing results indicate that bioaugmentation induced significant alterations in the bacterial community composition in BFS.

#### Conclusions

This study concludes that the presence of naturally occurring MSB significantly enhances the activity of cyanotoxin-degrading bacteria in BFS. The successful attachment and biofilm formation, as well as the increased degradation activity of bioaugmented bacteria, affirm the synergistic impact of MSB on the bioaugmentation process for the emerging contaminant removal.

#### Keywords

Biofilters, Bioaugmentation, Multispecies Biofilms, Cyanotoxins

## Impact of fungal pathogens and environmental conditions on the antimicrobial tolerance of *Pseudomonas aeruginosa* biofilms in the cystic fibrosis context

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### Background

Most current in vitro biofilm models to test the effectiveness of novel antimicrobials do not take into consideration the impact other cohabiting microbes and the CF environment on the effectiveness of the antimicrobials, resulting in clinical failure.

### Aims

To develop accessible polymicrobial biofilm models highly adaptable to CF conditions, and assess the impact other bacteria, fungi, media and oxygen levels have on the effectiveness of antibiotics against *Pseudomonas aeruginosa*.

### Methods

A polymicrobial colony biofilm model using relevant CF media, oxygen levels, bacterial and fungal species including *Pseudomonas aeruginosa*, *Staphylococcus aureus* and either *Candida albicans* or *Aspergillus fumigatus* was developed and treated with meropenem, colistin, tobramycin and DNase. Colony forming units and metabolic activity using a resazurin-based assay were used to determine the minimal biofilm inhibitory concentrations MBIC50 and MBIC90.

### Results

For *P. aeruginosa*, the MBIC50 and MBIC90 significantly increased when treated with meropenem and tobramycin in the polymicrobial setting but only the metabolic activity changed when treated with colistin. Increased tolerance was observed under anoxic conditions whereas the opposite happened when biofilms were pre-treated with DNase. Interestingly, treatment of *P. aeruginosa* polymicrobial biofilms resulted in a significant CFU increase of co-cultured microbes resulting from changes in population dynamics within polymicrobial biofilms. The MBIC50 and MBIC90 of meropenem, colistin, tobramycin and ciprofloxacin against *P. aeruginosa* were also significantly decreased when *A. fumigatus* was the fungal representative compared to *C. albicans* in the polymicrobial setting. The polymicrobial complexity of the biofilm model is being increased incorporating further CF relevant species including *Burkholderia multivorans* to assess their impact on antimicrobial testing as well as develop novel antimicrobial therapeutics.

### Conclusions

This work highlights the need to consider the polymicrobial nature and diverse range of CF environments for in-vitro antimicrobial testing against *P. aeruginosa* biofilms to maximise the success of treatment.

### Keywords:

polymicrobial, model-development, Cystic-fibrosis, fungal-biofilms.

## Biofilms colonizing stone heritage: the case of Assistenz kirkegård in Copenhagen.

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### **Background incl. aims**

Biofilms colonize any kind of surface and environment, including all those artefacts, monuments and sites with an artistic, aesthetic, ethnological or anthropological value that constitute our cultural heritage. Biofilms can be particularly problematic when the heritage piece is situated outdoors, because of limited or no possibilities of controlling climate, and thus the biofilms' growth. Because of biofilms' tolerance to chemical and physical stress, biocides are only partially and temporarily efficient, forcing conservators to repeat treatments with increasingly aggressive and toxic compounds.

Innovative approaches for biofilm removal based on a detailed knowledge of microorganisms' interaction with our heritage material are thus needed. In addition, simplified model systems to simulate these interactions in the laboratory are required to avoid testing new solutions directly on valuable heritage pieces.

### **Methods**

Here, we introduce a new longitudinal study (September 2023 to August 2024) to describe and monitor the evolution of bacterial and fungal biofilms colonizing the stone surface of six tombstones at the Assistenz Kirkegård in Copenhagen. Biofilms colonizing stones are known for being complex communities of both phototrophic and heterotrophic microorganisms, supporting each other metabolism and resilience to a broad variety of stress. With a conservation treatment in October 2023, the biofilm was removed from the six tombstones, but, nevertheless, is expected to grow again in the next year.

### **Results**

To identify the pioneer species and monitor the biofilm development, we are collecting samples for microbiome analysis just before the treatment and for a year after. In addition, pre-conservation samples have been processed for isolation of phototropic and heterotrophic microorganisms to then reproduce similar communities in the laboratory and investigate strategies for biofilm and fungal removal.

### **Conclusion**

The project is expected to shed light on the interactions among microorganisms responsible for degradation of our heritage, on the evolution of these communities and response to conservation treatments.

### **Keywords:**

conservation, stone heritage, communities, development

## G-quadruplex DNA - hemin interactions in Staphylococcal biofilms induce tolerance to hemin toxicity and electroactivity

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### Background

Bacterial biofilms possess different biochemical structures such as polysaccharides, proteins and nucleic acids which confer toughness, rigidity, elasticity, and protection to the biofilm cells. Recent findings have shown that bacterial biofilms have an abundance of non-canonical nucleic acid structures such as G-quadruplexes, however their physiological roles and survival advantage conferred on the bacteria remain unclear. In our previous works, we have observed G-quadruplex DNA structures interacting with hemin in Staphylococcal biofilms.

### Methods

Using *Staphylococcus epidermidis* models, cell viability assays and electrochemistry elucidated the role of G-quadruplex/hemin complexes in protecting against hemin toxicity to bacteria and mediating electroactivity. Differential pulse voltammetry (DPV) was carried out to determine the electrochemical signatures of extracellular DNA (eDNA) and non-eDNA mediated electroactivity in *S. epidermidis* biofilms. DPV was also used to characterize electroactivity of cells with extracellularly bound G-quadruplex DNA/hemin complexes. Chronoamperometry (CA) and immunological assays demonstrated that extracellularly bound multimeric G-quadruplex DNA/hemin complexes mediated electron transfer in both planktonic cells and biofilms. Immunolabelling and confocal microscopy of Indium Tin Oxide (ITO) electrodes with biofilm attached showed DNA-cell conformations on the electrodes.

### Results

Higher tolerance to hemin (5 – 200  $\mu\text{M}$ ) was observed in exopolysaccharide producing cells with extracellularly bound multimeric G-quadruplex DNA (5  $\mu\text{M}$ ). DPV signatures of DNA releasing, and DNA release-deficient mutants revealed that extracellular DNA mediates electron transfer and G-quadruplex/hemin complexes functioned as electron transfer mediators in biofilms. CA and immunological assays showed that extracellularly bound multimeric G-quadruplex DNA/hemin complexes enabled electron flow between *Staphylococci* cells and the external environment under low-oxygen conditions characteristic of biofilm infection sites. Electrical current range 0.05 – 0.08  $\mu\text{A}$  per 0.126  $\text{cm}^2$  electrode surface area was observed with *S. epidermidis* biofilms harbouring multimeric G-quadruplex DNA/hemin complexes and was higher than currents from biofilm without either G-quadruplex DNA or hemin or both. Confocal microscopy images of electroactive biofilm showed DNA connections between cells.

### Conclusions

These properties show that G-quadruplex/hemin complex can assist bacterial biofilms in hemin tolerance and extracellular electron transfer. This can be vital in gaining energy and cellular survival in low oxygen stress occurring in biofilm infections.

### Keywords:

bioelectrochemistry, bacterial electrophysiology, oxygen, ATP

## Maturity of *Pseudomonas aeruginosa* biofilms dictates the influence of host polymers on biofilm biomass, quorum sensing and virulence

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### Background

Biofilms are associated with most chronic infection and are often resistant to antibiotics and difficult to treat. To target and eradicate in vivo biofilms, it is crucial to understand physicochemical and biological characteristics of the biofilms in the host environment. Motivated by recent studies where host polymers altered antibiotic tolerance of pathogenic biofilms, here, we aim to investigate how host polymers, in particular hyaluronic acid (HA), affect biofilms depending on their maturity levels.

### Methods

PDMS disks (SYLGARD® 184, 40:1) were placed in 24-well plates. Bacterial suspension (*P. aeruginosa* mPAO1, OD<sub>600</sub>=0.05) in M9 media with/without HA (2 MDa, 2 mg/mL) was added to each well and incubated at 37 °C for biofilm formation. Biofilms were quantified using crystal violet (CV) staining and visualized by staining with SYTO9. The CV dye was eluted using 30% acetic acid and absorbance at 550nm was recorded. SYTO9-stained biofilms were analyzed by confocal laser scanning microscopy. RNA extraction from biofilms and qPCR were performed using RNeasy mini kits (QIAGEN) and iQ SYBR Green (Bio-Rad), respectively.

### Results

CV staining showed that HA negatively affected (40% reduction) the total biomass of biofilms grown for 72h. Similarly, smaller thicknesses (<10µm) were measured for biofilms cultured with HA compared to the control (ca. 20µm). Expression of quorum sensing (QS) genes was not affected by HA. However, formation of entropy-driven bacterial aggregations was confirmed in the presence of HA, which might have led to less biomass of surface-attached biofilms. Interestingly, expression of *toxA*, a virulence gene, was downregulated in the presence of HA (p=0.0002). To study the impact of HA on matured biofilms, 72h-old biofilms were prepared in M9 media followed by maturation in M9 with/without HA. Interestingly, delayed dispersion of biofilms was observed in the presence of HA (228% biomass at day 9), which is likely attributed to upregulation of genes involved in Las and Rhl QS systems. Suppression of *toxA* expression was observed in the presence of HA (p=0.04) similar to the 72h-old biofilms.

### Conclusions

HA affects QS, virulence, and biomass of *P. aeruginosa* biofilms depending on maturity levels of the biofilms.

### Keywords:

hyaluronate, *pseudomonas aeruginosa*, host-polymers, quorum-ensing

## Post-dispersion dynamics of biofilm: deciphering the fate of a disrupted biofilm matrix

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### Background and Aim

The detachment of biofilms often leads to its partial disintegration into fragments of extracellular polymeric substance (EPS) matrix, biofilm-associated cells, and detached biofilm clusters, occurring over a significant time scale. Comprehension of the underlying mechanism governing the relocalization efficiency of detached biofilm-associated cells and clusters, along with its impact on population dynamics in an established biofilm model, is impeded by inadequate research and a limited number of publications on this topic. The biofilm dispersion kinetics can be investigated by replicating the natural environmental conditions which contribute to dispersion events. The current study prioritizes unravelling the fate and relocalization efficiency of biofilm matrix components, biofilm-associated cells, and clusters after dispersion.

### Methods

The methodological approaches include assessing proportionality of live and dead cell populations using confocal laser scanning microscopy (CLSM) and flow cytometry. A qualitative examination of population dynamics was conducted using CLSM and advanced image analysis tools, were employed to monitor the discrepancy in morphological characteristics, spatial distribution of cells, and the integrity of the biofilm matrix. These techniques collectively provided insights into the fate, viability, and kinetics of the dispersed biofilm components.

### Results

A significant difference in the efficacy and viability of biofilm-associated dispersed cells as compared to detached biofilm clusters was observed. Dispersion events had noticeable impact on the relocalization efficiency of the dispersed entities, suggesting a potential disruption in the long-term dispersion kinetics. The comprehensive analysis of population dynamics revealed changes in both the structural and spatial arrangement of biofilm associated cells and clusters after dispersion along with alterations in the structural integrity, and chemical properties of the biofilm matrix. Additionally, a shift in the dispersion rate was observed, elucidating the velocity and effectiveness of the dispersion process under varying environmental conditions.

### Conclusions

The observed discrepancy in relocalization efficiency and population dynamics suggests potential consequences on microbial dispersion kinetics. The present study establishes the framework for further research on the impact of biofilm dispersion on microbial ecology in mixed culture biofilm models which have practical implications in several domains such as in biofilm-associated infections, healthcare settings, water treatment, and environmental science.

### Keywords:

Biofilm, Dispersion, Relocalization, Population dynamics

## How do interactions between lesser known bacterial cystic fibrosis pathogens and *Pseudomonas aeruginosa* affect sensitivity to antimicrobials?

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### Background

A large proportion of people with cystic fibrosis (pwCF) will suffer a lung infection with *Pseudomonas aeruginosa* (PA) in their lifetime. CF lung infections are polymicrobial and additional opportunistic pathogens include *Stenotrophomonas maltophilia* (SM) and *Achromobacter xylosoxidans* (AX). It is widely accepted that biofilm formation alters antimicrobial susceptibility. Interactions between PA and SM or AX in the context of antimicrobial susceptibility testing (AST) and within biofilms are not well understood. Here, we investigate changes to susceptibility, viability, metabolic activity and cell density of dual- and single-species biofilms.

### Methods

PA, SM and AX were tested alone or as co-cultures (PA + SM or AX).

Cefiderocol, meropenem, tobramycin and trimethoprim-sulfamethoxazole were tested against dual and single species cultures (EUCAST disc AST).

Dual or single species biofilms were established on polycarbonate discs on Muller-Hinton agar.

Biofilms transferred to antimicrobial containing MHA and incubated for a further 24-hours, disrupted and measured. Viability (colony forming units/mL), metabolic activity (A540/590nm) and cell density (A600nm) were determined.

### Results

Susceptibility of AX and PA in co-culture to cefiderocol was significantly higher and lower compared to single species respectively. Differences in susceptibility of the co-culture to meropenem, tobramycin and trimethoprim-sulfamethoxazole were measured but which were not significant.

PA and SM co-culture susceptibility to cefiderocol increased and decreased susceptibility compared with monocultures respectively. The susceptibility of the co-culture to trimethoprim-sulfamethoxazole was comparable to the susceptibility of PA alone but was significantly decreased compared to SM alone. Altered co-culture susceptibility in response to tobramycin and meropenem is found when compared to single species cultures but is not significant.

Single species biofilms exhibited lower metabolic activity when exposed to meropenem and ciprofloxacin compared to untreated biofilms; the only exception was PA biofilm exposure to meropenem where no difference was measured. Biofilm viability and cell density was reduced when biofilms were exposed to meropenem and ciprofloxacin.

### Conclusions

Susceptibility, viability, metabolic activity and density of dual species biofilms is altered compared to their single species biofilm counterparts. Characterising the effect of interactions on antimicrobial susceptibility will improve treatment courses and lead to personalised therapy for pwCF, better stewarding the use of antimicrobials.

### Keywords:

Cystic fibrosis, microbiota, antimicrobial resistance

## Modulating biofilm can potentiate the activity of novel plastic-degrading enzymes

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### Background

Plastic waste is a growing worldwide problem desperately requiring a solution. Only 9% of all plastic waste has been recycled, and although recycling rates are increasing, the cost of recycling and limited downstream uses of recycled plastic mean an alternative method that removes plastic is needed. The sudden introduction of waste plastics into our ecosystems over the last 120 years has prompted scientists to investigate if bacteria may have evolved the ability to degrade waste plastics. These investigations have led to the identification of a range of different bacterial enzymes that have been shown to degrade high prevalence environmental waste plastics. This highlights the potential of microorganisms as an enzymatic reservoir for prospective solutions to the plastic waste crisis. However, an issue that has arisen with many of the enzymes that have been identified so far is that their efficiency is too low, limiting their potential to be developed as plastic bioremediation tools. To this end many research groups are now focusing on modifying the enzymes to improve efficacy and stability or creating enzymatic chimeras that can be expressed in an autologous host. Here, we wanted to explore if modulating the levels of biofilm formation could increase plastic degrading efficiency.

### Methods

We use series of genetic constructs to influence the levels of the universal second messenger, Cyclic-di-GMP, to drive biofilm formation. We perform a range of plastic degradation assays to determine plastic weight loss or damage.

### Results

We hypothesised that enhancing a bacteria's ability to attach to and form a biofilm on plastic could maximise the local concentration of the enzyme around the target substrate, therefore increasing the overall rate of plastic degradation. We found that increasing biofilm levels, by manipulating the levels of the second messenger, Cyclic-di-GMP, led to increased levels of polyester degradation in cells expressing novel and well characterised plastic-degrading enzymes.

### Conclusion

This indicates that modulating biofilm formation is a viable mechanism to fast track the development of bacterial plastic bioremediation solutions.

### Keywords:

Biofilm regulation, disruption

## Staphylococcus spp forming biofilm like-intracellular bacterial communities

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### Background

Intracellular bacterial communities (IBCs) are biofilm-like structures living inside eukaryotic cells that protect bacteria from antibiotic treatment, the immune cells and serve as an intracellular reservoir in particular for urinary tract infections. Uropathogenic *Escherichia coli* (UPEC) is the main etiological agent, accounting for more than 80% of uncomplicated UTIs. During UTI, *E. coli* can adhere and invade the epithelial bladder cells, forming intracellular structures in the cytosol. IBCs can protect bacteria from antibiotic action and serve as an intracellular reservoir for recurrent UTI. Other bacteria genders and species, apart from *E. coli*, were identified in the cytoplasm of urinary epithelial cells from humans with UTIs. Some of these bacteria are: *K. pneumoniae*, and *P. aeruginosa*.

*Staphylococcus aureus* and coagulase negative staphylococci (CoNS) are members of the human skin and mucous membranes microbiota and classically were considered extracellular pathogens. But in the last decade, it has demonstrated its capacity to internalize into both phagocytic and nonphagocytic cells, and now is accepted as a facultative intracellular pathogen. Also, to a lesser extent *Staphylococcus haemolyticus* can cause UTIs.

### Methods

Here, we evaluated the presence of *Staphylococcus* spp. in urine of a healthy population and with symptoms of urinary tract infections. Urine was subjected to expanded quantitative uroculture. Intracellular bacteria were isolated by a centrifugation, antimicrobial treatment, cell lyses for release of bacteria and MALDI-TOF identification. Also, cells were stained for confocal microscopy visualization.

### Results and Conclusions

One hundred and forty-one people participated in the study. The gender distribution was 77 females (54.6%) and 64 males (45.4%). Sixty-nine belonged to the symptomatic group (48,9%) and 72 were healthy asymptomatic. Diverse species of *Staphylococcus* were isolated from EQUC that included *S. aureus*, *S. epidermidis*, *S. haemolyticus*, *S. lugdunensis*, among others. Interestingly, we were able to detect *Staphylococcus* intracellularly in 13.6% of 56 cases of non-symptomatics population. Up to our knowledge, it has not been described inside bladder cells. Moreover, new questions appear as healthy people have intracellular bacteria, most of them *Staphylococcus* spp.

### Keywords:

biofilm, intracellular bacterial communities, *Staphylococcus*

## The effect of probiotic cell-free supernatant and live biofilm on in vitro biofilm growth on pathogens isolated from urinary catheters

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Catheter-associated urinary tract infections (CAUTI) that occur in long-term catheterized patients may lead to severe, persistent infections or bacteremia. They are difficult to eradicate and control, as they are caused by multispecies biofilms that may have higher resistance to antibiotics. Recently, there has been a growing interest in using probiotics, like probiotic lactic acid bacteria, to battle against pathogenic biofilms. Here, a four-species in vitro biofilm model on catheters was developed, involving clinical strains of *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella oxytoca*, and *Proteus mirabilis* isolated from urinary catheters. And two probiotics, *Lactobacillus plantarum*, and *Lactobacillus rhamnosus* were used to obtain cell-free supernatant (CFS) and probiotic biofilms. We investigated the individual interactions between two probiotics and pathogen biofilms. Both *L. plantarum* and *L. rhamnosus* cultures grew well and lowered pH in 50%MRS for 48 hours. The pH of 48-h *L. plantarum*, and 48-h *L. rhamnosus* cultures decreased from 6.5 to approximately 4. Mono-species biofilm formation of all pathogens was inhibited in 25%, 50%, and 75% CFS, while only 10% CFS can lead to pathogen biofilm formation. That may be pH dependent. Furthermore, the absolute biomass of the four-species pathogen biofilm was higher than that of all mono-species pathogen biofilms through crystal violet quantification. What's more, *E. coli*, *P. aeruginosa*, and *K. oxytoca* were all killed in mono-species pathogen biofilms when 48-h *L. rhamnosus* biofilm existed. While *E. coli* and *K. oxytoca* were observed in the four-species pathogen biofilm. Thus, our study shows inhibitions of probiotic cell-free supernatant and live biofilm on pathogen biofilm growth and certain protection within four-species pathogen biofilm.

### Keywords:

biofilms, CAUTI, multispecies, probiotics, interactions

## The impact of spatial organisation and matrix components on bacteriophage infections in multispecies biofilms

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Bacteria are in constant risk of getting infected by viruses (phages). These infections can be lethal, introduce an array of auxiliary functions or disrupt existing genes and have great ecological and evolutionary impact. While bacteria mainly reside in structured biofilm communities consisting of multiple species, the majority of phage research has been conducted in planktonic cultures with a single bacterial host. To gain more insight into the dynamics of phage infections in the wild, we studied a synthetic community of *Escherichia coli*, *Kluyvera cryocrescens* and *Vibrio anguillarum* exposed to lytic phages. All three bacteria were manipulated to express distinct fluorescent proteins which enabled characterization of the spatial organisation by confocal laser scanning microscopy. Noticeably, *E. coli* was protected from phage exposure when mixed with and covered by the two other species, while almost being eradicated when growing on its own. Further investigation revealed that especially *V. anguillarum* ensured a high level of protection, and systematic deletion of biofilm-associated genes in this species uncovered that *rbmC* and *vpsMNOP* were important for the protection of *E. coli* upon phage exposure. To understand the protective mechanisms of these genes, we are currently testing if the matrix components directly interact with phages and influence phage diffusion and adsorption, or whether the reduced protection is an indirect effect, caused by changes in spatial organisation when matrix mutants are applied instead of wildtype *V. anguillarum*. This project is still ongoing, but our current conclusions are that community context matters for infection dynamics, in terms of spatial organisation, species diversity, and the matrix components produced.

### **Keywords:**

Spatial organisation, multispecies communities, bacteriophages

## Comparative physiological and transcriptome analysis of *Candida auris* and *Candida albicans* to exogenous homoserine-lactone

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### Background

Interaction between *Candida* species and *Pseudomonas aeruginosa* is frequently observed in clinical practice. The 3-oxo-C12 homoserine-lactone (3OC12HSL) is a well-known *P. aeruginosa* quorum-sensing molecule, of which effect is not fully understood for different *Candida* species. Therefore, we examined the effect of 3OC12HSL to *C. albicans* and *C. auris* virulence, biofilm formation and transcriptome in comparative manner.

### Methods

To reveal the molecular events induced by 3OC12HSL, a genome-wide transcript profiling was performed with *C. albicans* and *C. auris* culture following 100 $\mu$ M and 200 $\mu$ M 3OC12HSL exposure using total transcriptome sequencing (RNA-Seq). The effect of 3OC12HSL was assessed in planktonic and sessile growth-related experiments at 100 and 200 $\mu$ M 3OC12HSL concentration. An immunocompromised systemic mouse model was used to evaluate the effect of 3OC12HSL to virulence of *C. albicans* and *C. auris*.

### Results

The 3OC12HSL exposure –regardless applied concentrations– resulted 44 (i.e.: regulation of cell-cell adhesion, arginine metabolic process) and 76 (i.e.: mitotic cell cycle, chromosome localization, microtubule-based process) genes with at least 1.5-fold increase or decrease in transcription for *C. albicans*, while 45 (i.e.: fatty acid catabolic process, peroxisome) and 25 genes was observed with at least 1.5-fold increase or decrease in transcription for *C. auris*. Growth was significantly inhibited within 2 hours of the addition of 3OC12HSL in *C. auris*, but only the 200 $\mu$ M treatment had activity against *C. albicans*. Adhesion and biofilm formation were significantly inhibited by 100 and 200 $\mu$ M in *C. albicans*, while only the 100  $\mu$ M exposure had relevant negative effect on biofilm formation in *C. auris*. The 3OC12HSL treatment resulted statistically comparable fungal burden in kidneys compared to untreated control in *C. albicans*, while concentrations lower than 200 $\mu$ M caused a significant fungal burden decrease in *C. auris* compared to control.

### Conclusions

The difference in interaction between *C. albicans*/*C. auris* and *P. aeruginosa* 3OC12HSL may have relevant clinical impact. The ability of 3OC12HSL to change certain *C. albicans*/*C. auris* related characteristics (i.e.: virulence, adhesion, biofilm forming ability) may have useful tools to develop alternative antifungal strategies and support the understanding of fungal-bacterial pathogenic interactions.

### Keywords:

*Candida auris*, homoserine-lactone, biofilm, RNA-seq

## THE ROLE OF CAPSULE VARIANTS IN STAPHYLOCOCCUS HAEMOLYTICUS ON IMMUNE RESPONSE AND BIOFILM PRODUCTION

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### Background

*Staphylococcus haemolyticus* is a common cause of sepsis in premature babies and children with cancer. Aside from biofilm formation, capsule formation is one of the few traits associated with virulence that have been discovered in the *S. haemolyticus* genome. Only one capsule type has previously been described in *S. haemolyticus*, and our group has discovered five additional capsule types through pangenome analysis. Considering the role of the capsule in *S. aureus* virulence, adhesion, and establishment of biofilm, we would like to investigate whether the capsule plays a similar role in *S. haemolyticus*, and how this role might differ between different capsule types. We aim to investigate the association between capsule generation, biofilm formation, and virulence between clinical versus commensal *S. haemolyticus* isolates.

### Methods

To address our research questions, we will screen 25 different clinical strains using different in vitro approaches such as TEM imaging, in vitro biofilm formation analysis, proteomic analysis of the biofilm, and ex vivo blood survival assay. We will perform targeted mutagenesis on one capsule type and further investigate the immune responses against the *S. haemolyticus* ( $\Delta$ capA,  $\Delta$ capI) mutant and the WT in an ex vivo blood model.

### Results

A pangenome study has revealed five additional capsule types in our collection. We have selected 25 representative strains to characterize each capsule type and its biofilm ability. Since one capsule type is more prevalent in clinical strains, we have created isogenic mutants to look at this capsule type in more detail.

### Conclusions

Capsule virulence will be investigated in *Galleria Mellonella* and further by using a murine model to confirm if the capsule confers a higher virulence property than non-capsule-producing *S. haemolyticus* strains.

These results will enhance our comprehension of the phenotypic transition from capsular to biofilm states, providing a more detailed characterization of the process. In the end, this will also help to develop new therapeutic targets and improve treatment protocols for *S. haemolyticus* infections in immune-compromised patients.

### Keywords:

Biofilm, CoNS, Capsule, Virulence, Clinical-strains

## Outer membrane vesicles as a mechanism of bacterial communication in uropathogenic biofilm-producing bacteria.

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One of the most common infections in humans are urinary tract infections (UTIs). *Escherichia coli* (Ec) and *Proteus mirabilis* (Pm) are the principal pathogens responsible for UTIs. Gram negative bacteria are capable of producing outer membrane vesicles (OMVs). These OMVs can act as a bacterial delivery system, by providing the possibility to export DNA, RNA, lipids, proteins, and toxins, among others. The OMVs could participate in biofilm production, nutrients acquisition, and as immune protection, being the target of several antimicrobials and phages.

The aim of this work was to isolate OMVs from previously characterized clinical isolated Ec\_U144 and Pm2921 in Luria-Bertani broth (LB) and artificial urine (AU), and to evaluate their possible function. OMVs were purified by filtering and ultracentrifugation. The OMVs were characterized on size and superficial charge by DLS (Zetasizer ZS), and imaging by TEM (STEM Inspect F50). Bacterial suspensions (OD<sub>600</sub>/0.5) of Ec\_U144, Pm2921 and Ec ATCC were incubated for 30 min, 1, 3, and 5 h with FM4-64-labeled OMVs in order to evaluate the association of OMV with bacteria. The association rate was obtained by quantification of the acquired bacterial fluorescence observed after the incubation. Finally, the effect of OMVs over biofilm formation was evaluated by the violet crystal method.

The Ec\_U144 OMVs have a size of 185 and 257 nm in LB and AU, respectively. Meanwhile, the Pm2921 measured 267 and 320 nm. All OMVs have a negative zeta potential. Related to the OMV association, Ec\_U144 was able to accept its own OMVs after 30 minutes incubation, while Pm2921 takes more than 3 hours to accept their own OMVs. Interestingly, Ec ATCC was able to accept both OMV from Ec\_U144 and Pm2921 in 30 min and 3 h respectively. The difference of association observed could imply roles between the OMVs, once probably for intraspecies and the other for interspecies communication. When we evaluate the biofilm formation, we observed a significant increase in biomass when Ec\_U144 and Pm2921 were incubated with their own OMV. We conclude that Ec and Pm produce OMVs that participate in communication and enhance biofilm formation.

### **Keywords:**

UTI, OMV, Communication, Biofilm

## Corticosteroids fluticasone propionate and budesonide affect virulence and biofilm formation in *Pseudomonas aeruginosa* regardless of its source

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### Objectives/aim

Corticosteroids (CS) fluticasone propionate and budesonide are commonly used to reduce airway inflammation in respiratory diseases, being the key stone in the treatment of the Chronic Obstructive Pulmonary Disease (COPD). However, high doses of CS have been associated to an increased incidence of *Pseudomonas aeruginosa* (PA) infections in these patients. Previous work from our laboratory revealed that the CS affected biofilm formation of the type strain PAO1. However, there is no information regarding how CS might affect PA virulence-related traits in strains from different sources. Thus, our goal was to investigate how CS affect PA strains from different origins in terms of motility, pigment production and biofilm formation at 24/48h.

### Materials/Methods

The PA strains included were: ENV1&2 (environmental), BSI1&2 (bloodstream infections isolates), COP2&6 (COPD isolates) and PAO1 (control). Motility patterns (swarming, swimming and twitching) and determination of pigments pyoverdine and pyocyanin were performed following standard procedures. Biofilm was prepared using the cfp-labelled version of the strains for confocal imaging at 24/48h starting by a OD<sub>600</sub>=0.1 normalised culture (in RPMI). Treatment consisted of 1µM of CS fluticasone propionate or budesonide (high dose used in the therapeutic range) or EtOH (solvent control).

### Results

All strains responded differently to the corticosteroids. The most affected traits were pyoverdine production and biofilm formation, although no common patterns were found among the strains of the same source. In general, an increment of pyoverdine production correlated with an increased biofilm biomass at 24 or 48h. CS had no impact on biofilm on 2 strains, ENV2 and COP2. Interestingly, our previous work showed that COP2 increased its biofilm biomass in response to CS when co-cultured with the lung cell line A549.

### Conclusion

CS could contribute to the pathophysiology of PA infections by affecting biofilm formation among other virulence features. However, the differences in the observed effects suggest that the genetic background of the strain might be of importance. Further studies are guaranteed regarding the mechanisms behind these observations, and we hope the genomic analysis of the studied strains (currently ongoing) will shed some light in this regard.

### Keywords:

*Pseudomonas aeruginosa*, corticosteroids, biofilm, virulence

## Development of a functional 3D model of lung cells to study the interaction of *Histoplasma capsulatum* biofilms

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*Histoplasma capsulatum* exhibit the ability to undergo morphological transformation in response to environmental while transitioning from the environment to the host system. This intracellular pathogen, which can form biofilms in vitro, is known to initiate respiratory infections in mammals with progress to systemic infections. While research efforts have predominantly focused on host-pathogen interactions using 2D cell models, the literature extensively covers a spectrum of 3D models elucidating host-pathogen interactions in various microorganisms, however, research on dimorphic fungi remains limited. In this context, our research aimed to develop a 3D functional human lung tissue model as an infection model to elucidate the mechanism of host-pathogen interaction in *H. capsulatum* biofilms. To achieve this, we developed a 3D human lung tissue model through the co-cultivation of differentiated lung epithelial, fibroblasts, and macrophage-like cells (THP-1) at Air-Liquid Interface (ALI). Collagen type I hydrogels were employed as the scaffold material in a proportion of 3:3:1. The model viability was assessed using the XTT assay and quantification of total carbohydrates over a period of 28 to 30 days post ALI differentiation. Furthermore, *H. capsulatum* biofilm-derived infections within the 3D model were visualized using Fluorescence Microscopy (FM). Human collagen type I was used due to its potential to reduce immunogenic reactions, thereby minimizing interference with host-pathogen interactions involving *H. capsulatum*. Subsequent validation of our model, assessed through cell viability using the XTT method and total carbohydrate quantification, revealed an intriguing pattern. We observed an increase in metabolic activity and higher sugar consumption between the 7th and 14th days, as well as between the 9th and 19th days of model development. Significantly, this phenomenon coincided with the period of ALI cultivation of lung epithelial cells. Examination of infection results through FM images unveiled notable filamentation of *H. capsulatum* in both planktonic and biofilm-derived cells. A transition from the yeast to the mycelial phase of the fungus was evident. The biofilm-derived infection remarkably demonstrated more extensive filamentation compared to planktonic yeast infection. Our results suggest that these factors, coupled with the physical barrier presented by the 3D model, likely contributed to hyphae formation.

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### **Keywords:**

*Histoplasma capsulatum*, 3D-lung model, biofilm

## Benzodiazepines modulate *Pseudomonas aeruginosa* biofilm formation

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### Background/aim

*Pseudomonas aeruginosa* (PA) is one of the main opportunistic nosocomial pathogens, being the main cause of ventilator-associated pneumonia (VAP) in ICU patients, with high morbi-mortality rates. Benzodiazepines (BZ) are used as a sedative during intubation/ventilation, but constitute a risk factor for VAP due to undesirable effects on the patient. On the other hand, there is evidence that PA could show receptors capable of detecting and reacting to BZ. Our objective was to determine if the exposure of PA to physiological concentrations of BZ could modulate its behaviour in terms of biofilm formation.

### Methods

PAODK biofilm formation in response to BZ was assessed by Cristal Violet staining (CV) following standard procedures, and further investigated by confocal microscopy (CM, cfp-labelled strain normalised at OD<sub>600nm</sub>=0.1, RPMI-1640) for 24h. eDNA release in response of BZ was also determined by CM (0.5 µM of Di-YO-1, 10' staining). Biomass (cells or eDNA) was quantified with COMSTAT. c-di-GMP production (biofilm global regulator) was monitored using a transcriptional reporter (PAO1\_PcdrA::lux). BZs diazepam or midazolam at 10 µg/ml (serum concentration achievable in ventilated patients) or 100 µg/ml (simulating a potential cumulative effect of sedatives inside biofilms) compared with controls without drug or with the solvent (40% propylene glycol, 10% ethanol, 4.9% sodium benzoate, 0.1% benzoic acid) were used.

### Results

CM revealed a significant increase of biofilm biomass and eDNA release in response to 10 µg/ml of both BZ, ca. 60% for bacterial cells and eDNA, while no differences were found by CV. Conversely, at 100 µg/ml, CV revealed that both BZ significantly increased the total biofilm biomass (up to 100% for midazolam). BZ also incremented c-di-GMP production compared to the solvent control at 10 µg/ml. Conclusion. Exposure to BZ can affect PA biofilm formation, which could contribute to the success of PA to cause VAP in intubated and BZ-sedated patients, as the increase of adhesion/biofilm formation on the endotracheal tubes used for such procedures would constitute a bacterial reservoir.

### Keywords:

*Pseudomonas aeruginosa*, benzodiazepines, biofilm modulation

PG1-37

### Impact of DMEM Conditioning on Microbial Adherence and Biofilm Characteristics in Endodontic Infections

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#### Background

Dulbecco's Modified Eagle Medium (DMEM) has emerged as a promising alternative to conventional simulated body fluids for assessment of the bioactivity of biomaterials and understanding their in vivo behaviour. However, the effect of conditioning surfaces with DMEM on microbial adherence and biofilm characteristics in endodontic infections remain unexplored.

#### Aims

This study aimed to investigate the impact of preconditioning 12-well plates or hydroxyapatite (HA) coupons with DMEM on subsequent microbial adherence and associated characteristics of mono- and dual-species biofilms of *Enterococcus faecalis* and *Candida albicans*.

#### Methods

Mono and dual-species biofilms of *E. faecalis* and *C. albicans* were cultured in 12-well plates, either DMEM preconditioned or non-conditioned. Metabolic activity, biomass, and adhered cell count were measured using AlamarBlue, crystal violet, and microbial cell enumeration, respectively. Temporal effects of DMEM conditioning on biofilm development were studied, with and without media replenishment. Additionally, the impact of DMEM preconditioning on gene expression of microbial surface proteins was investigated. Dual-species biofilms were also grown on HA coupons conditioned with DMEM or water for 24 h in a CDC biofilm reactor. Confocal laser scanning microscopy and Live/Dead® BacLight™ bacterial viability were used to assess biomass, average thickness, and roughness coefficient of biofilms.

#### Results

DMEM conditioning did not significantly alter microbial adherence or biofilm biomass on 12-well plates. However, molecular changes were observed. After 1 h adherence on DMEM conditioned surface, *E. faecalis* showed non-significant upregulation of *efaA* and *epal*. However, at 72 h significant downregulation of these genes was observed. *Candida albicans* on DMEM-conditioned surfaces exhibited significant downregulation of only *als3* at 24-h and both *als1* and *als3* at 72-h. On HA coupons, DMEM conditioning led to reduced live biomass and increased roughness coefficient of dual-species biofilms.

#### Conclusion

Our findings suggest that DMEM conditioning influences gene expression related to adhesion and certain characteristics of biofilms associated with endodontic infections. This study provided insights into DMEM's impact on microbial behaviour and biofilm formation.

#### Keywords:

conditioning surfaces, adherence, biofilm, endodontic

## Pathogenic Effects of *Pseudomonas aeruginosa* With Different Levels of Cyclic Diguanosine Monophosphate in Pulmonary Infection of Rats

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### Abstract

**Background** *Pseudomonas aeruginosa* (*P. aeruginosa*) is regulated in many ways by the Cyclic diguanosine monophosphate (c-di-GMP) signaling pathway system. But the regulation of c-di-GMP on the pathogenicity of *Pseudomonas aeruginosa* remains unclear. In this study, animal models were established to explore the pathogenicity of different levels of c-di-GMP strains in rats with pulmonary infection of *P. aeruginosa*. **Methods** The agarose inclusion of *P. aeruginosa* PAO1/plac-yhjH strain, PAO1 strain and PAO1 $\Delta$ wspF strain were injected into the trachea of the rats through tracheotomy. The content of bacteria in the lungs on the 1st, 3rd and 5th day after the operation was detected. The pathological changes of lung were observed by hematoxylin-eosin (HE) staining. The levels of tumor necrosis factor (TNF)  $\alpha$  in serum and lung tissues were determined by enzyme-linked immunosorbent assay (ELISA). **Results** PA was detected in the lung tissue of the three groups on the 1st, 3rd and 5th day after inoculation. The bacterial counts of the lung tissue, the severity of inflammation of the lung pathology, the severity of inflammation of the lung pathology, the expression of TNF- $\alpha$  in serum and the expression of TNF- $\alpha$  in lung tissue homogenate of rats from high to low were as follows: group W > group P > group Y ( $P < 0.05$ ). **Discussion** Inoculating *P. aeruginosa* with different levels of c-di-GMP, the bacteriology of lung tissue, degree of lung tissue damage, inflammation of the lung, expression of TNF- $\alpha$  are as follows: group W with high level of c-di-GMP > group P with normal level of c-di-GMP > Group Y with low level of c-di-GMP. These results suggest that intracellular c-di-GMP signaling molecules are involved in the pathogenic process of PA pulmonary infection, and the level of c-di-GMP is correlated with the virulence of bacteria.

### Keywords:

*Pseudomonas aeruginosa*; rat model; infection

## Evaluating the Biofilm Formation of *Pseudomonas aeruginosa* PAO1 Against Ciprofloxacin in an Artificial Infection Model at J774A.1 cell line under Aerobic and Anaerobic Conditions: Apoptosis-Necrosis and Nitric Oxide Responses

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### Aims

Artificial infection models are prepared in aerobic versus anaerobic conditions to analyze the laboratory side with a translational perspective. Virulence characteristics of *Pseudomonas aeruginosa* including biofilm and antibiotic resistance are important to be searched in terms of human health.

### Methods

Minimum inhibitory concentrations (MICs) of biofilm producing *Pseudomonas aeruginosa* PAO1 (*P. aeruginosa* PAO1) against ciprofloxacin were found under aerobic conditions (aeroMIC, 50% aeroMIC) and anaerobic conditions (AN-aeroMIC, 50% AN-aeroMIC). Then J774A.1 cells were infected with *P. aeruginosa* PAO1 (w/o Ciprofloxacin)[m.o.i: 50:1] and incubated against MICs and 50%MICs of Ciprofloxacin under aerobic and anaerobic conditions (Anaerobic: 10% hydrogen, 10% carbon dioxide, 80% nitrogen) conditions. Apoptosis/necrosis and nitric oxide (NO) responses of cells, and biofilm formations were evaluated at the 3rd, 6th and 18th hours. Results: The aeroMIC of *P. aeruginosa* PAO1 was 0.062 µg/mL while AN-aeroMIC was 2 times higher at 0.125 µg/mL. *P. aeruginosa* PAO1 formed biofilm in aerobe and anaerobe conditions at 18th hours ( $p < 0.05$ ). Biofilm was formed under 50% aeroMIC and AN-aeroMIC effect at the 18th hour. Ciprofloxacin suppressed NO responses of infected cells under aerobic and anaerobic conditions in all experimental groups including MICs and 50%MICs at 3, 6, 18th hours. NO response of anaerobic control (w/o) was higher than aerobic control (w/o) at 6th hour. At 18th hour NO response was higher in aerobe control (w/o) than anaerobe control (w/o) whereas AN-aeroMIC NO value was higher than aero-MIC value, and NO value of aero-50%MIC was higher than AN-aero50%MIC. The necrosis rate of cells infected with *P. aeruginosa* PAO1 control under aerobic conditions were found to be higher than in anaerobic conditions at 3rd and 6th hours ( $p < 0.05$ ). It was determined that a higher rate of necrosis response developed under aerobic conditions in infected cells compared to anaerobic conditions at 18th hour ( $p < 0.05$ ). Conclusions: As a result, we may conclude that *P. aeruginosa* showed different pathogenesis and resistance pattern to ciprofloxacin in anaerobic versus aerobic environments, and it seems that oxygen-dependent experimental models will be inspiring, especially in discussion of post surgical infections where the risk of biofilm formation is higher and treatment options are confusing.

### Keywords:

Biofilm, Aerobic and Anaerobic Conditions

## Bacterial biofilm mineralization on dental composite: an in vitro model.

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### Background

Bacteria in the oral cavity form dental plaque, which may mineralize and become calculus. Dental plaque and calculus are associated with dental diseases such as caries and periodontitis. Due to the pathological relevance, various in vitro approaches have been proposed to characterize the formation of oral biofilms albeit failing to create a model universally accepted by the dentistry community. Here, we follow an original method to study the calculus formation on dental composites using a model organism in a tailored bioreactor that mimics the mouth environment.

### Methods

The bioreactor for oral biofilm mineralization stems from a model used to investigate cariogenic processes. To simulate humid (but not submerged) growth conditions, samples of dental composites previously inoculated with E.coli W3110 were placed in a chamber with continuous drop-wise supply of nutritious media. Two different media were used: a control medium with nutritious substances and a mineralizing medium consisting in control medium supplemented with mineral precursors. Both the dental composite sample and the bioreactor designs resulted from an optimization process thoroughly documented in this work. To locate the bacteria using Confocal Laser Scanning Microscopy (CLSM), we used an E.coli W3110 strain producing mCherry fluorescent proteins in their cytoplasm; Calcein Green and Calcofluor White marked the presence of mineral and biofilm extracellular matrix (ECM), respectively.

### Results

CLSM provided an overview of the bacterial tissue in conditions close to the native state. All the samples showed bacterial and ECM presence, but only in samples grown in the mineralizing medium minerals were observed. Scanning electron microscope (SEM) pictures of dried and fixed samples showed biofilm at the bacteria length-scale. The samples incubated in the control medium had a thinner biofilm layer; while the biofilm cultivated in mineralizing medium was much thicker with conglomerates that hint towards mineralization.

### Conclusions

We achieved an in vitro model representative of the conditions of growth and mineralization of biofilms in the mouth: something between the traditional solid-air and solid-liquid interface models. Such model will be useful for systematic studies on various dental substrates.

### Keywords:

Biofilm, Mineralization, Dental Calculus, Bioreactor

## Biofilm formation of *Phaeobacter piscinae* on pro-bacterial polymer surfaces

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Fish production in aquaculture is increasing dramatically to provide food for the growing world population. To control bacterial infections under intensive rearing conditions, antibiotics have often been used leading to fish pathogens becoming resistant to antibiotics. Therefore, new sustainable solutions to prevent fish diseases must be found and one option is using probiotic bacteria. Bacteria of the Roseobacter group have been tested as probiotics in marine larval rearing, and their production of tropodithietic acid (TDA) enables pathogen control. This secondary metabolite, produced by several *Phaeobacter* species, has antimicrobial properties, and its production appears enhanced when the bacteria grow in biofilms compared to planktonic cultures. The purpose of this project is to develop a method that allows comparison of biofilm formation and TDA production measured by proxy using gene expression of a core TDA biosynthetic gene operon, *tdaCDE*. *Phaeobacter piscinae* was allowed to form biofilm on polypropylene coupons placed in microtiter plates containing 3% Instant Ocean, 0.3% casamino acids and 0.2% glucose. The surfaces were transferred daily to fresh media. For biofilm formation analysis, the surfaces were rinsed with water and stained with Syto62 to monitor the biofilm biomass. GFP expressed from the promoter of the *tdaCDE* operon was used to track the TDA biosynthetic gene expression. Images of the biofilm were acquired with a confocal laser scanning microscope, and the biovolume and gene expression were then quantified using the software BiofilmQ with the Otsu method as a threshold. Biovolume increased from 4.42  $\mu\text{m}^3/\mu\text{m}^2$  at 48 h to 8.13  $\mu\text{m}^3/\mu\text{m}^2$  at 72 h ( $p=0.0009$ ). Also, *tdaC* gene expression increased, albeit not statistically significant ( $p=0.2251$ ). No correlation was detected between *tdaC* gene expression and biovolume ( $p=0.0502$ ) as *tdaC* gene expression per biovolume was higher at 48 than at 72 h. Our ongoing experiments aim to explore how different surface topographies and characteristics affect biofilm formation and *tdaC* expression, and the ultimate goal is to design surfaces allowing efficient probiotic activity.

### Keywords:

Roseobacter, TDA, Polymer surfaces

## Bronchial epithelial cell Air-Liquid-Interface (ALI) biofilm model for improved antimicrobial susceptibility testing in *Staphylococcus aureus* biofilm-related lung infections

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### Background/Aims

Current in vitro biofilm antimicrobial susceptibility testing (AST) methods have low predictive value for anticipating treatment outcomes of biofilm-associated infections. This deficiency is largely ascribed to the absence of physiological microenvironments reflective of in vivo biofilm formation. Our bronchial epithelial Air-Liquid-Interface (ALI) *Staphylococcus aureus* biofilm model aims to mimic more closely chronic lung infections, where the biofilms integrate as small aggregates into the mucus. This model is predicted to better reflect microbial physiology and hence antimicrobial susceptibility.

### Method

For ALI cultures, human primary bronchial epithelial cells were cultured on the polyester membrane of both 24- and 96-well transwells, positioning their basal surfaces in contact with liquid media while exposing the apical side to air. Media was changed for 28 days until the development of a mucociliary pseudostratified epithelium, confirmed by FITC-dextran permeability assays and fluorescent antibody-staining of mucus-producing goblet cells and ciliated cells. Subsequently, ALIs were either infected with planktonic cells or preformed biofilm microclusters of GFP-labelled MRSA ST-239. Biofilm formation as well as the effect of antibiotic treatment was observed at different time points via confocal laser scanning microscopy and CFU determination.

### Results

Biofilm formation was reproducibly observable in both 24- and 96-well ALI set-ups as clusters around the lung cells. Biofilms developed faster on biotic surfaces compared to the abiotic surfaces of conventional microtiter plates (MTP). First antibiotic susceptibility testing with the same concentrations of trimethoprim/sulfamethoxazole plus rifampicin resulted in higher reduction of biofilm cell numbers in the ALI than MTP model. No toxic effect on the lung cells was observed.

### Conclusion

Our bronchial epithelial ALI *S. aureus* biofilm model may pave the way for an innovative, standardized, physiologically relevant diagnostic tool for biofilm AST in clinical microbiology laboratories. The scalability of this model to 96-well formats, coupled with the integration of rapid, label-free readout techniques such as optical coherence tomography (OCT) or fluorescent lifetime imaging microscopy (FLIM), may render it suitable for high-throughput screening of anti-biofilm compounds against chronic lung infections in the future. Clinical trials evaluating potentially superior in vivo antimicrobial susceptibility predictability of our novel ALI-biofilm model compared to MTP models are urgently needed.

### Keywords:

air liquid interface, biofilm diagnostics

## A novel chronic wound infection model to study *P. aeruginosa* virulence factor AaaA

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### Background

*Pseudomonas aeruginosa* poses a formidable challenge in healthcare settings due to its intrinsic multi-drug resistance, leading to persistent infections in immunocompromised individuals. This significant global disease burden is particularly evident in the context of cystic fibrosis, where *P. aeruginosa* is a primary cause of fatal lung infections, and in chronic wound infections where it frequently coexists with other bacteria. Our study focuses on unravelling the functional intricacies of a key virulence factor, the arginine-specific aminopeptidase of *P. aeruginosa* (AaaA), which is crucial for the establishment and persistence of chronic infections. Understanding AaaA's role is pivotal, as it could potentially serve as a target for vaccines or antimicrobials.

### Methods

To realistically mimic host infection sites, a synthetic chronic wound model incorporating *Staphylococcus aureus* alongside *P. aeruginosa* was developed. Lab strains (PAO1L and SH1000) and paired clinical isolates were used. Confocal microscopy was conducted after 1, 2, 4, and 8 days incubation and then the models were homogenised to isolate the bacteria for the AaaA specific activity assay and CFUs.

### Results

Quantification of AaaA activity in the lab strain models demonstrated a time-dependent increase, while a mutant lacking *aaaA* exhibited significantly reduced L-arginine cleavage within just 2 days. However, in models containing the clinical isolates a surge in AaaA activity was seen after day 1, with AaaA activity plateauing from day 2 onwards. Confocal microscopy highlighted distinct differences in biofilm formation between the *aaaA*-defective mutant and the wild type after 4 days in the lab strains. However, this difference was more subtle with the clinical isolates.

### Conclusions

Our findings support the hypothesis that AaaA plays a crucial role in biofilm formation, explaining its significance in chronic compared to acute infections. The enhanced model aims to provide insights into how AaaA could be targeted effectively in a polymicrobial context, offering valuable implications for the development of antimicrobial strategies. Further research is planned to explore the role evolutionary co-culture has on AaaA function. This will be conducted by mismatching the clinical isolate pairs to establish if the role of AaaA in a polymicrobial context is affected by congruent growth and development.

### Keywords:

Chronic wound, virulence factor, dual-species

## Designing structures to support growth of intestinal biofilms in a colonic in vitro model

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The gastrointestinal (GI) tract is host to a complex community of microbes existing within different niches, including free-flowing planktonic microorganisms, and microbial biofilms within the intestinal mucosal layer. Biofilms within the colon are thought to play a key role in triggering inflammatory mechanisms through direct contact with host cells or via microbially produced metabolites. However, , given the difficulties studying these communities in situ, our understanding of colonic biofilms remains limited. Therefore, we propose a platform that can accurately replicate these complex biofilms in vitro, supporting research of the GI tract sessile populations.

Structures to support biofilm growth were designed to integrate a clinically reflective in vitro model of the human colon, called MiGut. The structures were 3D printed in biocompatible resin and featured 12 removable coupons to allow for biofilm sampling. We investigated 2 coupon designs, the first featuring a plain surface and another featuring a 1.3mm honeycomb pattern. Experiments were performed using 4 MiGut reactors, 2 of which contained a plain biofilm structure and 2 with the honeycomb structure. MiGut models were inoculated with human faeces (10% w/v) to replicate the human microbiota and populations were allowed to stabilise/form biofilm for 14 days. Biofilm coupons and planktonic samples were then collected for DNA extraction and real-time quantitative PCR (qPCR) analysis. 2 coupons from each structure were washed and stained with 0.5% (w/v) crystal violet. The biofilm biomass was then determined spectrophotometrically at OD600.

Similar DNA concentrations were observed on both coupon types and qPCR showed an increase in Bifidobacterium, Eubacteria, and Prevotella when using the honeycomb design compared to plain. The crystal violet staining revealed that biomass adhered to all coupons, with absorbances of 0.364 AU and 0.855 AU for the plain and honeycomb designs respectively.

These results show that the structures are able to support colonic biofilm formation within MiGut, and that specific design choices of the coupons can improve biofilm yield. Future work will focus on incorporating mucin to represent the mucosal environment and to investigate changes in biofilm composition over a period via the use of multiple sampling points.

### **Keywords:**

Gut microbiota, in vitro, chemostat

## Development of an Innovative in vitro Prosthesis Joint Infection- model for Staphylococcus aureus biofilm study

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Biofilm formation on orthopedic implants is often implicated in chronic prosthesis and joint infections (PJI) that are complex to manage. To date, no current bacterial in vitro model can fully recapitulate the PJI context leading to a lack of knowledge to develop diagnosis tool and adapted treatment.

Our project aims to set up an innovative in vitro model to characterize Staphylococcus aureus clinical strains biofilms in a PJI context. Biofilm formation was evaluated in various culture media and by counting both planktonic and adherent bacteria (CFU) and quantifying biofilm biomass using crystal violet staining.

In this work, we have optimized the parameters of this model in terms of time of incubation (24, 72 or 96h). A mature biofilm was obtained after 72 hours of incubation with a similar proportion of planktonic and adherent bacteria whereas a variable dispersion was observed at 96.

We compared two different oxygen concentrations (Hypoxia 2% in bone site vs Anoxia) and concluded that even a slight variation had an impact on bacterial growth, adhesion, and biomass underlining the fact that the physiological conditions are necessary to consider for a mimetic model. A physiologically medium has been developed for this model, the modified Bone-Like Environment (BLE+), with a 5-fold-reduction of nutrient concentration but 10-fold increase of Mg<sup>2+</sup> compared to the reference (Minimum Media). BLE+ led to the development of a thicker biofilm compared to the reference media (MM).

Lastly, to avoid the sedimentation of aggregates that can be present in vitro, we used titanium structures suspended from the cover and specifically created for this approach. Preliminary results have shown that biofilms from MRSA or MSSA vary in biofilm formation in this specific PJI model.

In conclusion, we successfully validated parameters for an in vitro biofilm model simulating PJI context. These parameters include 2% dioxygen, BLE+ supplementation, and 72-hours incubation on suspended titanium pegs. Currently, we are exploring various clinical S. aureus strains to validate our model. This research aims to uncover PJI-specific biofilm behaviors, enabling the refinement of therapeutic strategies tailored to effectively combat PJI.

### **Keywords:**

Staphylococcus aureus, Biofilm model, IOAP

## Paracoccidioides brasiliensis biofilm: Extracellular Matrix, Glucan Synthesis, Associated Genes and Adhesins.

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### Background

Paracoccidioides encompasses the Paracoccidioides brasiliensis complex, consisting of four phylogenetic species. A distinguishing feature between planktonic growth and biofilm is the presence of a 3D extracellular matrix (ECM). In this study, we examined biofilm formation in the P. brasiliensis (Pb18) yeast phase, characterized the structural elements of the matrix, and evaluated the expression of glucan genes at different stages of biofilm evolution. Methods. The strain was cultured in planktonic and biofilm forms for 24 to 144 hours. Fungal biomass and metabolic activity were assessed using crystal violet and tetrazolium salt reduction (XTT) tests, and colony-forming units (CFU) were quantified through plating. Biofilm structure was visualized using scanning electron microscopy and confocal laser scanning microscopy techniques. The extracellular matrix of P. brasiliensis biofilms was extracted by sonication, and polysaccharides, proteins, and extracellular DNA (eDNA) were quantified. RNA was extracted with the Trizol<sup>®</sup> reagent, quantified, and then used for cDNA synthesis. Enolase expression and the genes 14-3-3, FKS1, AGS1, GEL3, and KRE6 were analyzed using real-time PCR. Results. The studied strain of Paracoccidioides formed biofilms with significantly higher metabolic activity and biomass values at 144 hours. The extracellular matrix of P. brasiliensis had a higher content of polysaccharides, followed by proteins and smaller quantities of eDNA. The L34 gene emerged as the most effective normalizer. In the quantitative assessment of glucan expression in P. brasiliensis under biofilm conditions, the expression of GEL3 and enolase genes increased within the initial 24 hours. Throughout the biofilm maturation phase, an upregulation was observed in the expression of 14-3-3, AGS1, and FKS1. Additionally, a decrease in KRE6 expression and an increase in GEL3 were noted at 144 hours. Conclusion: This study underscores the biofilm-forming potential across P. brasiliensis and emphasizes the key constituents of the extracellular matrix. These findings contribute to a more comprehensive understanding of biofilm organization and aid in identifying potential targets for in-depth investigation and assessment of the applicability of anti-biofilm therapy.

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### Keywords:

Paracoccidioides brasiliensis. Biofilm. ECM. Adhesins.

## Galleria mellonella larvae as a model for studying implant-associated biofilms and MBEC analysis for *S. aureus* and *E. faecalis* biofilms

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### Background

Implant-associated biofilms caused by *S. aureus* and *E. faecalis* are challenging to treat due to antimicrobial tolerance. Invertebrate models, such as the *Galleria mellonella* larvae model, are being explored due to ethical constraints on mammalian models. However, implant-associated biofilms have rarely been analyzed in the *G. mellonella* model. The study aimed to evaluate different methods of forming biofilms on medical implants in larvae to mimic implant-associated biofilms. Additionally, the study aimed to assess treatment efficacy using the standard antibiotic treatment regime for prosthetic endocarditis.

### Methods

Biofilm formation on the implant inside the larvae (IL) and pre-formed biofilm on the implant (PF) implanted in the larvae were the two approaches compared. The implant was a cardiovascular suture composed of polytetrafluoroethylene (ePTFE) cut into 0.5 cm in length. Three clinical isolates of each *Staphylococcus aureus* and *Enterococcus faecalis* with a standardized dose of  $10^5$  CFU/larvae were used. In the first group, the implant was implanted one hour before the larvae were infected with  $10^5$  CFU/Larvae through proleg. In the second group, the implant was pre-incubated with bacterial suspension and implanted into the larvae through the proleg. Biofilm formation was confirmed through scanning electron microscopy. Both biofilm methodologies were also compared for the evaluation of antibiotic treatment regimes such as the combination of vancomycin and rifampicin.

### Results

Both implant groups, compared to the non-implanted (planktonic infection) group, exhibited lower larval survival rates due to hypermelanization against bacterial attachment on the implant, resulting in bacterial spread in the hemolymph. Pre-infected implants showed higher survival rates than the in vivo biofilm method, despite both methods resulting in  $10^6$  CFU bacterial adhesion to the implant. The formation of biofilm was confirmed by scanning electron microscopy. The combination of vancomycin and rifampicin caused a 5 log<sub>10</sub> reduction in CFU count of the pathogen.

### Conclusion

Overall, the *G. mellonella* larvae model was proven to be suited for studying implant-associated biofilms and showed the capability of assessment of qualitative (biofilm visualization) and quantitative (CFU count) parameters. In the future, this model can be used to evaluate the efficacy of novel antibiofilm compounds and anti-adhesive surface-modified implants against biofilms.

### Keywords:

Implant biofilm, *Galleria mellonella*, antibiotics

## Galleria mellonella larvae: a promising animal model to study biofilm maturation in orthopedic infections

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### Introduction

The development of biomaterial-associated infections (BAI) is one of the most common complications affecting trauma patients, requiring prolonged hospitalization and the intensive use of antibiotics. Following the attachment of bacteria on the surface of the biomaterial, the biofilm-forming bacteria could initiate a chronic implant-related infection. Despite the use of conventional local and systemic antibiotic therapies, persistent biofilms involve various resistance mechanisms that contribute to therapeutic failures. The development of in vivo chronic BAI models to optimize antibiofilm treatments is a major challenge because of the biofilm pathogenicity and the challenging regulation of the host response.

### Aim

Previously, a *Galleria mellonella* larvae model for the formation of an early-stage biofilm on the surface of a Kirschner (K)-wire was established. In the present study, two models of mature biofilm using clinical *Staphylococcus aureus* strains were assessed: one related to contaminated k-wires (in vitro biofilm maturation) and the second to hematogenous infections (in vivo biofilm maturation). Rifampicin (80 mg/kg) was used as a standard drug for antibiofilm treatment.

Methodology: In the first model, biofilms were formed following an incubation period (up to 7 days) in a CDC Biofilm Reactor (CBR, BioSurface Technologies). Then, after implantation of the pre-incubated K-wire in the larvae, rifampicin was injected and the larvae survival was monitored. In the second model, biofilm formation was achieved after an incubation period (up to 7 days) inside the larvae and then, after removing the K-wires from the host, in vitro rifampicin susceptibility assays were performed (EUCAST).

### Results and conclusions

The first model indicates that in vitro biofilm maturation affects the bacterial pathogenicity in the host, depending on the *S. aureus* strain. Furthermore, the more the biofilm is mature; the more rifampicin treatment efficiency is compromised. The second model shows that, despite the fast in vivo biofilm formation time in the host, the number of bacteria attached to the k-wire surface and in the larvae tissues were not increasing over time. Altogether, these results allow the establishment of biofilm models using *G. mellonella* larvae in order to understand the impact of biofilm maturation on both the bacterial pathogenicity and the efficiency of antibiofilm treatments.

### Keywords:

in vivo, biofilm maturation, rifampicin

## Unfolding genotypic interactions in a synergistic multispecies community

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### Background

The strains *Paenibacillus amylolyticus*, *Microbacterium oxydans*, *Stenotrophomonas rhizophila* and previously, *Xanthomonas retroflexus*, were co-isolated from the soil residuesphere and shown to possess synergistic biofilm formation capabilities in vitro. This model community has potential industrial applications, such as providing drought resistance to plants and enhancing the degradation of waste products like keratin. We aimed to conduct a bioinformatic analysis of these isolates and identify certain genomic features that can be linked to the social interactions we observe in vitro.

### Methods

The genomes of the 4 strains were hybrid assembled, using both long and short reads. The top gene functions were characterized using PATRIC subsystems, KEGG, and GO term classifications. Other analyses, such as construction of a phylogenetic tree including related, common species, identification of secondary metabolite gene clusters, and specifying the types of antimicrobial resistance genes, were conducted. We also utilized the newly released tool, SOCfinder, to identify social genes in our genomes.

### Results

After assembling the circularized genomes, we found that the previously regarded *X. retroflexus* was instead *Stenotrophomonas maltophilia*. When comparing the 2 *Stenotrophomonas* isolates, they had an average nucleotide identity (ANI) of 85%. The top gene function categories for *S. rhizophila* and *S. maltophilia* were two-component system, ribosome, and oxidative phosphorylation, while ABC transporters, two-component system, and purine metabolism/ribosome were the top categories for *P. amylolyticus* and *M. oxydans*, respectively. *P. amylolyticus* had 16 secondary metabolite gene clusters identified in its genome, while the other 3 strains all had 2 clusters. When identifying social genes, *P. amylolyticus* again came out to have the greatest number of cooperative genes, followed by *S. maltophilia*, *S. rhizophila*, and finally, *M. oxydans*.

### Conclusions

Overall, analysis of the complete genomes of this model community provides insight to the positive social interactions observed in vitro, as well as uncovers major gene functions which could play a role in the observed community intrinsic properties.

### Keywords:

Multispecies biofilms, genomics, social microbiology

## Biofilm target attainment of amoxicillin and benzylpenicillin in patients with infective endocarditis

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### Background

Infective endocarditis (IE) is considered a biofilm infection of the endothelial surface of the heart.  $\beta$ -lactams play a central role in the antibiotic treatment of IE. The time above MIC of the free unbound concentration ( $fT > MIC$ ) is correlated to efficacy, but the specific clinical pharmacokinetic/pharmacodynamic (PK/PD) target in IE remains debatable. Higher plasma levels for an extended period of time may be required in biofilm infections due to increased tolerance and resistance.

### Methods

Pharmacokinetic measurements were performed in 116 patients treated with oral amoxicillin (1000 mg q6h) and 75 patients treated with intravenous benzylpenicillin (3000 mg q6h) for left-sided IE in the Partial Oral Endocarditis Treatment (POET) trial. Population PK modeling was performed to estimate the probabilities of target attainment (PTAs). In addition, model-based simulations were conducted for benzylpenicillin, evaluating different MIC values and various PK/PD targets.

Results. For amoxicillin, the PTA with a standard PK/PD target of 50%  $fT > MIC$  was 100% for streptococci and staphylococci, and 96.8%-100% for enterococci. Using a target of 50%  $fT > 4 \times MIC$  the PTA was 93.3%-100% for streptococci and staphylococci, and 77.4%-96.0% for enterococci. However, using a clinical breakpoint (BP) for enterococci of 4 mg/L the PTA with a target of 50%  $fT > 4 \times BP$  was only 18.3%-18.9%.

For benzylpenicillin, the PTA with targets of 50%  $fT > MIC$  and 50%  $fT > 4 \times MIC$  was 96.4%-100% for streptococci and staphylococci, and only 0%-22% for enterococci. Using targets of 100%  $fT > MIC$  and 100%  $fT > 4 \times MIC$  the PTA was 71.4%-100% for streptococci and staphylococci, and 0% for enterococci. Model-based simulations showed that high PTAs ( $> 90\%$ ) were achieved for MIC values  $\leq 0.5$  mg/L with a standard target of 50%  $fT > MIC$ , and for MIC values  $\leq 0.063$  mg/L with targets of 100%  $fT > MIC$  or 50%  $fT > 4 \times MIC$ .

### Conclusions

The majority of patients with IE attain the target levels of orally administered amoxicillin and intravenously administered benzylpenicillin, particularly those infected with streptococci or susceptible staphylococci. However, caution is warranted when treating IE caused by enterococci with amoxicillin or benzylpenicillin. Further studies are required to assess the penetration and distribution of  $\beta$ -lactams in IE vegetations to ensure attainment of the PK/PD target at the site of infection.

### Keywords:

Endocarditis Pharmacokinetics Pharmacodynamics Amoxicillin Benzylpenicillin

## Developing and validating an automated high-throughput sub-gingival biofilm model

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### Background

Multiple devices have been described as tools for studying oral biofilms in vitro, however few have the automated and high-throughput capacity to study sub-gingival plaques in depth. Sub-gingival biofilms are crucial in the pathogenesis of periodontal disease with poorly understood mechanisms for transition from benign to harmful. The in vivo position and variation make these microbial communities particularly challenging to research, so the requirement for models capable of high-throughput cultivation in vitro with advanced environmental parameter control is highly desirable. Described here is a model that is adaptable and scalable through modular design. Our model features independently controlled modules designed to mimic the environment below the gumline.

### Methods

Our system has been tested using a previously validated 5-species consortium comprised of bacteria implicated in the pathogenesis of periodontal disease. Biofilms on hydroxyapatite discs were harvested following 2-weeks of automated operation by mechanical disruption and quantified through agar enumeration.

### Results

4 modules were employed, exhibiting different environmental conditions comparing stirred/unstirred, static/constant media flow, and two substrate densities. Analysis of the microbial abundance from all biofilms showed that *P.gingivalis* and *P.intermedia* were the highest colonisers in the biofilms, whilst *S.salivarius* was the lowest. In comparing stirred versus unstirred environments, aerobes *S.salivarius* and *A.naeslundii* showed increases in CFU/mL, indicated by log changes of 0.477 and 0.156, respectively. In contrast, gram-negative anaerobes experienced decreases, with *P.gingivalis* showing the largest decline (-0.186 log). Similarly, in the transition from static to constant media, these aerobes increased (log changes ranging from 0.252-0.577 for *S.salivarius* and 0.686-0.783 for *A.naeslundii*). while *P.intermedia* exhibited the most significant decrease among remaining species, with up to -0.514 log change depending on substrate density.

### Conclusions

Our platform has demonstrated that biofilms can be cultured anaerobically whilst maintaining constant flow and stirring, which is more reflective of the in vivo sub-gingival environment. This proof-of-concept experiment has demonstrated similarities in periodontal microbial ecology with increasing environmental complexity offered with our advanced model. Our model could benefit periodontal research by offering a versatile platform which balances accessibility, clinical relevance, and experimental efficiency, promising insights into sub-gingival biofilm dynamics.

### Keywords:

Sub-gingival, automated, high-throughput, continuous flow



## Exploring secrets of biofilm and biopolymer production in an engineered system: Genomic diversity of Zoogloea

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### Background

Biofilms are ubiquitous and crucial in various environments, yet they often remain understudied, particularly in environmental and engineered systems with their microbial producers being poorly described. Among these, wastewater treatment plants (WWTPs) stand out as ideal model systems for investigating microbial biofilms and flocs with Zoogloea as a key organism, serving as a valuable subject for studying extracellular polymeric substances (EPS) biosynthesis. In the RETHiNk project, we aim to utilize EPS from WWTPs to produce sustainable biopolymers, replacing oil-based polymers. However, gaps in our understanding of EPS composition, formation, and microbial producers must be addressed before commercialization. This study aims to uncover this through molecular studies of Zoogloea as a model organism, shedding light on its functional and evolutionary relationships within the genus.

### Methods

Recent discoveries have highlighted the importance of two extracellular polysaccharide (exoPS) gene clusters, comprising 7 and 28 genes, in Zoogloea resiniphila for floc formation. Through genome-mining we investigated the conservation of these exoPS systems across the Zoogloea genus. Additionally, we mapped the distribution throughout the bacterial kingdom to understand the extent of exoPS production conservation. To validate the structure and conservation of exoPS production, EPS has been extracted from three in-house isolates (*Z. caeni*, *Z. resiniphila*, and *Z. oleivorans*) which are to be used for chemical and structural analyses to verify genomic findings.

### Results

The two gene clusters were found to be conserved in the entire Zoogloea genus. Two clades of the 7-gene gene cluster within the Zoogloea genus were observed, suggesting differences in exoPS biosynthetic pathways even within the genus. The gene clusters were exclusively detected in the Burkholderiales order and mainly in the Rhodocyclaceae family. However, they were not consistently detected in phylogenetic restricted groups (genera/families/orders), meaning species-level differences within genera, families and orders exist.

### Conclusions

Besides being conserved in the entire Zoogloea genus, the two Zoogloea exoPS gene clusters are widely distributed in the Rhodocyclaceae family suggesting its critical role in biofilm/floc formation. Mapping the phylogenetic distribution of EPS biosynthesis systems is crucial for predicting the production of EPS in microbial systems. This is especially significant when aiming to recover these components.

### Keywords:

Biopolymer, wastewater treatment, genomics, genome-mining

## Observing bacterial-fungal interactions in a realistic chronic wound model and their tolerance towards common clinical treatments

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### Background

Chronic wounds are a continuous burden on healthcare systems, and their global occurrence is predicted to increase. A hallmark of chronic wounds is the presence of microbes, which cause high amounts of tissue damage and delay healing. Microbes are often found in chronic wounds in the biofilm lifeform, negatively impacting treatment options due to biofilm properties, such as increased antimicrobial tolerance. Wounds have been found to contain several different bacterial species, along with fungi and viruses. Recent studies suggest that interkingdom and interspecies interactions may occur and cause an increased tolerance towards treatments. Such interactions complicate the infections but are rarely represented in in vitro wound models. Therefore, the aim was to create a realistic in vitro wound model where these interactions could be investigated.

### Methods

In this study, we established an interkingdom, tri-species chronic wound model containing *Candida albicans*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*. The model contains mammalian-derived components solely, such as whole blood, serum, fat, and meat digest and is solidified using collagen. The models were either left untreated or treated with a combination of bandages, antibiotics or antifungal compounds. Interkingdom and interspecies interactions were investigated by colony-forming units (CFU) on selective plates and visualized using CLSM. Moreover, changes to the models' microenvironment were tracked by measurements of pH and oxygen using microsensors.

### Results

Preliminary data showed that growth profiles from single-, dual- and tri-species models differed, suggesting that the 3 species affected each other when growing together. Moreover, the tolerance towards different clinical treatments, such as silver-infused bandages and antibiotics, was altered in the tri-species models compared to the single- and dual-species models.

### Conclusion

Preliminary data suggests that interspecies interactions were present within the models, leading to varying tolerance profiles depending on the residing microbes. Our results suggest that co-infected wounds containing fungi and bacteria may need altered treatment regimens to account for interkingdom interactions.

### Keywords:

in vitro, interactions, fungi, treatments

## Assessing the impact of *Staphylococcus aureus* in planktonic and biofilm states on wound healing in a murine model

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### Background

Biofilms have been strongly implicated in delayed wound healing. There is however uncertainty about the relative contributions of bacteria in the planktonic versus biofilm phenotypes, to delayed healing. This emphasises the need to examine the differences in wound healing process between planktonic and biofilm-infected wounds through preclinical models. Wound dressings often contain antimicrobial compounds, notably silver-based ones, but their effectiveness against planktonic or biofilms varies.

### Method

A time-course in vivo excisional wound murine model was developed to facilitate an understanding of the mechanisms of wound biofilm formation. Wounds were challenged with preformed *S. aureus* biofilms or with similar cell numbers of planktonic cells of same bacterium. Then, the efficacy of silver oxynitrate dressing, which produces highly reactive Ag<sup>2+</sup> and Ag<sup>3+</sup> ions, was evaluated. Wound healing was characterised by quantification of wound area, and re-epithelialisation from histological sections. Inflammation was determined based on neutrophil/macrophage marker immunohistochemistry. Wound biofilms were characterised by tissue Gram stain, quantitative bacterial culture, and scanning electron microscopy.

### Results

Over 7 days, wound width and areas increased significantly in wounds challenged with preformed biofilm compared to planktonic cells. A significant reduction in the reepithelialisation percentage was observed in wounds challenged with preformed biofilms compared to planktonic cells. In contrast, uninfected wounds exhibited a comparatively small wound width, area, and comparatively high reepithelialisation percentage. The number of neutrophils and macrophages was significantly higher in wounds challenged with biofilms. *S. aureus* abundance on the surface of both planktonic and biofilm challenged wounds was markedly increased compared to uninfected wounds. Silver oxynitrate dressings significantly reduced *S. aureus* viable counts, wound area, inflammation, and increased reepithelialisation more in planktonic challenged wounds.

### Conclusion

The murine wound models provide evidence of the impact of *S. aureus* biofilm form in delaying healing process compared to planktonic form.

### Keywords:

Planktonic, Biofilm, Murine model

## In vitro simulation model for infective endocarditis shows pathogen-specific response

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### Background

Infective endocarditis (IE) is a life-threatening infection of the inner layer of the heart. IE is considered a biofilm infection and treatment consists of long-term antibiotic combination therapy according to guidelines. However, lethality of IE remains high. New treatment strategies need extensive in vivo testing, which can be limited due to ethical, economical and work-load reasons. Therefore, we developed an in vitro model to resemble biofilm infected vegetation tissue. This allows for easier testing of antibiotic combinations before advancing to animal testing and clinical trials.

### Methods

IE vegetations (IEVs) in patients are constituted by fibrin, thrombocytes, leukocytes and bacteria. The IEV simulation model is produced by collecting whole venous blood into specific devices (3C© system), followed by centrifugation in a custom centrifuge (REAPPLIX, DK). The resulting patches consisting of three distinct layers (fibrin, thrombocytes, leucocytes) are divided into 6mm biopsies and are inoculated with *Staphylococcus aureus*, *Streptococcus mitis*, *Enterococcus faecalis* or *Pseudomonas aeruginosa*. Biofilm development was assessed after different timepoints via quantitative bacteriology, confocal laser microscopy and tolerance development towards relevant antibiotics. Antibacterial properties of the IEVs were measured by chemiluminescence (respiratory burst) and Luminex (cytokine production).

### Results

We demonstrated the presence of bacterial microcolonies on the model by confocal laser microscopy six hours after infection. Biofilm development was also supported by antibiotic tolerance towards 10x MIC concentrations of tobramycin, ciprofloxacin and benzylpenicillin. We furthermore demonstrated that the patch exhibited distinct respiratory burst activity and cytokine release after *Staphylococcus aureus* vs *Pseudomonas aeruginosa* infection (e.g. IL-1b increase of 3.8-fold vs. 27-fold). Our results have led to an ongoing clinical study investigating IEVs from the blood of IE patients together with their IE-causing bacterial isolate.

### Conclusion

We have established an in vitro simulation model for IE that could potentially mirror the ongoing IE in patients and lead to a more personalized choice of antibiotic treatment. The model can furthermore be used to test novel antibiotic strategies for IE.

### Keywords:

Infective endocarditis, simulation model, biofilm

## Evaluation of the in vitro anti-biofilm activity of oral products containing cetylpyridinium chloride (CPC) and/or O-cymen-5-ol

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### Background

Prevalent oral diseases such as gingivitis and periodontitis are associated with bacterial biofilms. For their control, the use of chemical hygiene complements such as mouthwashes and dentifrices is commonplace. Objective: The aim of the study was to evaluate the antimicrobial efficacy of mouthwashes and tooth pastes on an in vitro multispecies biofilm model. Methods: The biofilms were prepared from a suspension containing:  $10^3$  CFU/mL of *Streptococcus oralis* and  $10^6$  CFU/mL of *Fusobacterium nucleatum*, *Aggregatibacter actinomycetemcomitans* and *Porphyromonas gingivalis* each. To evaluate the antimicrobial activity of different oral hygiene products on the formed biofilm, the 5-5-cyano-2,3-ditoly tetrazolium chloride (CTC) assay and confocal laser scanning microscopy (CLSM) with SYTO<sup>®</sup> 9 + propidium iodide, were used. Results: The different products evaluated in this model of biofilm gave similar results to previous analyses with a different model (*Streptococcus mutans* and *Prevotella intermedia* instead of *S.oralis* and *A. actinomycetemcomitans*). Both the viability test by CTC and CLSM showed that the addition of both products as well as their combinations in oral products is effective in reducing biofilm. Conclusion: The new model serves to evaluate emerging oral products that potentially could improve oral health and help to fight against oral biofilm. The addition of essential oils seems to be useful as active ingredient in oral care products.

### Keywords:

Mouthwashes; Toothpaste; Oral biofilm control.

## A GelMA-based in vitro model to mimic Staphylococcus aureus growth and biofilm formation in tissues

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### Background

Biofilm research has grown exponentially over the last decades, providing key insights into the mechanistic behavior of bacterial communities, however little of the basic research in labs has been translated to actual therapeutic approaches. This low bench-to-bedside translatability is caused by the use of over-simplistic in vitro models that overlook the physiological conditions where biofilms thrive in vivo, the properties of the host microenvironment, as well as the biofilm-host interactions. In this study, we use gelatin methacryloyl (GelMA), which has a longstanding track record in serving as an extracellular matrix proxy for a variety of tissues, to study *S. aureus* growth and biofilm formation under physiologically relevant conditions.

### Methods

*S. aureus* were embedded in 5%, 10% and 15% GelMA hydrogels with Young's modulus ranging between 1-100 kPa and their growth was followed over 21 days. Confocal microscopy was used to follow population changes using Live/Dead (Syto9/PI) staining and matrix deposition using DAPI/ConA. In addition, bacterial viability and metabolic activity were assessed using CFU counting, and PrestoBlue assays. Finally, AFM and microindentation were used to investigate mechanical changes undergone by GelMA constructs as a result of bacterial growth and biofilm formation.

### Results

Confocal microscopy revealed that earlier in the growth path, *S. aureus* formed heterogeneously distributed dense clusters. These clusters were significantly higher in size in softer gels (5% > 10% > 15% GelMA) and continued growing over time until forming a layer of bacterial cells with secreted matrix components towards the edge of the constructs after 14 days in the case of 5% GelMA and 21 days for 10% and 15% GelMA systems. Interestingly, bacteria embedded in 10% and 15% GelMA had a significantly higher metabolic activity in comparison to the softer gels, suggesting a key role to mechanical cues, nutrient gradients and water content in *S. aureus* growth and biofilm formation.

### Conclusions

We demonstrated that biofilms grown in a GelMA-based 3D microenvironment show a dynamic structure and growth in x, y, and z dimensions, proposing a more relevant in vivo-like architecture in comparison to standard biofilms grown traditionally on 2D substrates at solid/liquid interfaces. Finally, this model can be further developed to study the tissue/implant interface in implant-related infections.

### Keywords:

Biofilm, In vitro model, Infection

## Etiology of joint prosthesis biofilm infection in Uruguay from 2019 to 2023

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### Background

Bacterial infections in prosthetic joints pose a significant postoperative complication, leading to high morbidity and mortality rates. These infections, occurring predominantly within the first two years post-implantation, extend antibiotic use, prompt surgical interventions, and elevate healthcare costs. Microorganisms forming biofilms on orthopedic implants contribute to persistent infections, defying patient immune responses and prophylactic treatments. In Uruguay, between 2019 and 2023, 5% of joint replacements were due to infections out of 6467 primary hip prosthesis implants. This study aimed to detect and identify microorganisms forming biofilms on prostheses, providing insights into joint prosthesis infections in Uruguay.

### Methods

Biofilm presence on removed infected implants was determined using the sonication technique, with crystal violet staining for macroscopic observation. A pilot test explored the use of resazurin for detecting metabolically active biofilms on laboratory-generated samples.

### Results and

From October 2019 to December 2023, 46 hip joint prostheses were analyzed, revealing a 76% recovery of microorganisms from biofilms. *Staphylococcus aureus* and *S. epidermidis* were most frequently identified (22% and 25%, respectively), along with *E. faecalis* (11%), *Corynebacterium* spp (8.5%), *K. pneumoniae* (5.7%), and *P. aeruginosa* (5.7%). Fungal presence was also noted in 8.5% of prostheses, highlighting the complexity of mixed biofilms. The study assessed different culture media for in vitro biofilm formation, with BHI+Glu proving effective. Clinical isolates demonstrated enhanced biofilm formation in more restrictive media. DMEM also supported biofilm formation. Preliminary trials with resazurin showed promise for evaluating metabolically active biofilms in situ, though further testing on recently removed prostheses is needed.

### Conclusions

In conclusion, this study identified common microbiological agents causing prosthetic replacements due to biofilm formation in Uruguay. It explored various biofilm cultivation conditions and assessed resazurin as an alternative detection method, offering valuable insights for developing strategies to reduce infection rates and effective treatments for bacterial biofilms.

### Keywords:

joint prosthesis, etiology, biofilms, Uruguay,

## Validation of the antimicrobial effect of tranexamic acid using in vitro and in vivo models

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### Background

Recent in vitro research showed that tranexamic acid (TXA) alone and in combination with vancomycin and gentamicin had a synergistic effect against a few staphylococcal strains. In the present study, this synergistic effect was validated on samples from patients with Staphylococcal periprosthetic infection in addition to an in vivo model.

### Methods

We tested 19 clinical strains (5 *Staphylococcus aureus*, 14 coagulase negative Staphylococci [CoNS]) against 10 mg/ml TXA alone and in combination with serial dilutions of vancomycin and gentamicin. The standardized microtiter plate method was used. Minimal inhibitory concentrations (MIC) were calculated using standard visualization of well turbidity. We also tested in a *S.aureus* (ATCC29213) PPI murine subcranial model the synergistic effect of TXA and gentamicin in comparison to TXA or gentamicin alone after 4 days of monitorization. Then, discs were removed for cfu/ml counts and cell viability rate. Biofilm structure of both in vitro and in vivo samples was also analyzed by scanning electron microscopy (SEM).

### Results

When TXA was combined with vancomycin or gentamicin, a reduction of MIC was demonstrated in 30% of the strains studied. According to species, MIC<sub>50</sub> for vancomycin and gentamicin alone and in combination with TXA against *S.aureus* strains were the same. In the case of CoNS, the same occurred with vancomycin and its corresponding combination, whereas with gentamicin and TXA, a 2-fold reduction in MIC<sub>50</sub> was observed (fig.1a). In addition, in the in vivo model, the mean log cfu/ml and cell viability rate obtained from the implant was lower in the group of mice treated with TXA and gentamicin compared to those treated only with TXA or gentamicin. SEM images also corroborate our findings in those strains in which MIC was reduced, as well as the in the mice implants, being higher the biofilm occupation area in those samples treated only with gentamicin or TXA compared to those treated with TXA+gentamicin (fig.1b).

### Conclusions

We confirm that TXA in combination with vancomycin or gentamicin does have a synergistic effect by reducing bacterial aggregation, however, it only occurs in selected strains.

### Keywords:

TXA, vancomycin, gentamicin, murine model.

## Anti-biofilm characterization of PEEK coated with antiseptics.

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### Background

The capacity of microorganisms to generate a biofilm on implants' surface is higher in hydrophobic polyether ether ketone (PEEK) prostheses compared to metal materials. The biofilm is an aggregation of microorganisms that can occur on any surface, being able to generate an extracellular matrix that confers a barrier against both the host's immune system and the action of antibiotics. This usually implies the removal of the prosthesis, which has important clinical and economic consequences. It is therefore necessary to search for preventive and therapeutic alternatives that allow the surface of the materials to be modified in order to reduce bacterial adherence. We aimed to improve in an in vitro model the prophylactic anti-biofilm capacity of PEEK by coating with antiseptics such as menthol (M) and povidone iodine (PI).

### Methods

We used 6 mm in diameter and 3 mm in height PEEK discs previously sterilized that were coated either with 4 mg/ml M solution in 2% DMSO during 5 min or 24h (under agitation) or with PI during 1 min (positive controls were only treated with DMSO 2% or sterile saline). Then, discs were washed with PBS and inoculated with bacterial suspensions (0,5 McFarland) during 24h at 37°C under agitation. Microorganisms tested were the following: *Staphylococcus aureus* ATCC29213, *Staphylococcus epidermidis* (clinical strain), *Enterococcus faecalis* ATCC35186, *Pseudomonas aeruginosa* ATCC27853, *Candida albicans* ATCC14053. Discs were then washed with PBS and sonicated to calculate log cfu/ml by serial dilution cultures (fig.1a). We calculated log cfu/ml percentage reduction of each experiments as follows:  $[1-(\log \text{cfu} \times \text{ml}^{-1} \text{ coated-peek} / \log \text{cfu} \times \text{ml}^{-1} \text{ peek})] \times 100$ .

### Results

Overall, both in M and PI experiments, there was a minimal reduction in bacterial growth compared to the positive control, with the highest values being obtained for only *P.aeruginosa* at M 5 min, M 24h, and PI 1 min, respectively: 15.2%, 14.2%, and 13.8% (fig.1b).

Conclusions: We failed to demonstrate an anti-biofilm effect of coated PEEK discs (log cfu/ml reductions were <20%). Further studies are needed to optimize the penetration of antiseptics by impregnation techniques that allow to modify the hydrophobicity of PEKK material.

### Keywords:

peek; biofilm; menthol; device-related infections.

## OTHER

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### Biofilms of ciprofloxacin-resistant *P. oleovorans* recovered from automatic washer-disinfectors (AEWD) for reusable medical devices in comparison to *P. aeruginosa* reference strain

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#### Background

Outbreaks resulting from contaminated endoscopes are due to inadequacies in the reprocessing procedures. Repeated controls of AEWDs have consistently identified the presence of *Pseudomonas oleovorans* in two separate endoscope reprocessing departments within our hospital. The bacterium was repeatedly found despite the automated chemo-thermal disinfection process.

#### Methods

Contact cultures were taken from predilection sites in the washing machine. We typed representative isolates using RAPD-PCR. Antimicrobial susceptibility testing was performed according to EUCAST. Planktonic and biofilm forms of both *P. oleovorans* strains and a *P. aeruginosa* reference strain (Pa01) were exposed to 1% Neodisher Septo DN2 (20g Glutaral in 100g) both at 55°C as per the manufacturer's recommendation and at 25°C for 5 minutes. Efficacy against biofilms was evaluated according to ASTM E2799-17 using the Minimum Biofilm Eradication Concentration (MBEC) assay and the XTT-assay for metabolic activity. All tests were repeated six times.

#### Results

RAPD-PCR distinguished two different strains of *P. oleovorans*, one of which was resistant to ciprofloxacin. The planktonic forms of both *P. oleovorans* strains were easily eradicated by exposing them to the disinfectant according to manufacturer's recommendations. *P. oleovorans* formed significantly weaker biofilms than *P. aeruginosa* (OD600: 0,028/0,068 vs. 0,791). There was no significant decrease in biofilm metabolic activity 2 hours after exposition to 1% disinfectant at 55°C for 5 min. At 25°C, the disinfectant killed *P. oleovorans* biofilms only at a concentration of 4%.

#### Conclusion

The survival of pathogenic bacteria in biofilms could be promoted by using an inappropriate temperature or disinfectant concentration. Although there were no cases of *P. oleovorans* infections, it should be kept in mind that ineffective disinfection can lead to iatrogenic transmission and infections, especially in high-risk patients such as the immunosuppressed.

#### Keywords:

*P.oleovorans* , biofilms, disinfection, endoscope

## Characterisation of biofilms found in poultry and pig farm drinking water systems

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### Background

In the context of the general pigs and poultry health, drinking water quality is often overlooked as a source of infection. The quality depends on microbial load and chemical composition, which can be improved through various cleaning and disinfection or water treatments. However, persistent biofilms in drinking water systems can lead to re-contamination resulting in lower water quality. Understanding the microbiological composition of biofilms found in pig and poultry houses is a first crucial step for addressing this issue.

### Methods

Biofilm swab samples were collected from the interior surface of the pipelines of the drinking water systems near the drinking nipples, across 15 broiler farms and 15 pig nursery units. The sampling occurred at the end of a production round before cleaning and disinfection. Microbiological enumerations and chemical analyses were performed on these. The dominant bacterial flora (n=1000 isolates) present on the total aerobic count (TAC), pseudomonas agar base and coliform plates were selected and purified followed by identification through Maldi-TOF and 16S rRNA sequencing. Finally isolates, selected based on cluster analysis, were investigated for their biofilm-forming capacity on microtiter plates through crystal violet staining and classified as weak, intermediate, or strong biofilm formers.

### Results

Results showed a comparable biofilm presence in both animal primary production sectors with average counts of TAC, pseudomonades, and coliforms of  $3.5 \pm 0.9$ ,  $2.9 \pm 0.8$  and  $1.8 \pm 0.8$  CFU/cm<sup>2</sup> respectively. Predominant bacterial groups included staphylococci (18%), pseudomonades (13%), and microbacteria (7%). Key species identified were *Pseudomonas fluorescens* (5%), *Staphylococcus cohnii* (5%) and *Stenotrophomonas maltophilia* (4%) present in the biofilm across 23 of the 30 farms. Among the 213 selected isolates, 78% exhibited biofilm-forming capacity, with 28% classified as strong biofilm formers. All *Pseudomonas fluorescens* (n=14), *Staphylococcus cohnii* (n=7) and *Stenotrophomonas maltophilia* (n=9) strains demonstrated biofilm formation, with strong biofilm formation amongst respectively 43%, 30% and 90% of the isolates.

### Conclusions

The strong biofilm-forming capacity of key species like *Pseudomonas fluorescens*, *Staphylococcus cohnii*, and *Stenotrophomonas maltophilia* emphasizes the need for better prevention measures to mitigate re-contamination and enhance overall drinking water quality in pig and poultry houses.

### Keywords:

Biofilm, water, microbiology, pigs, poultry

## Effects of saliva and serum on eubiosis and dysbiosis in multispecies oral consortia

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<sup>1</sup>Malmö University, Malmö, Sweden

### Background

The oral microbiome plays an important protective role by resisting colonization by human pathogens. However, it also underlies oral diseases such as periodontitis, which affect around 1 billion people worldwide. In health, a complex balance with the host is responsible for maintaining a healthy (eubiotic) microbiota but environmental factors can shift this equilibrium, leading to dysbiosis in microbial communities and disease. One such factor is a change in nutrient availability at the gum margin due to increased flow of gingival crevicular fluid (GCF) in gingivitis. To better understand the transition from eubiosis to dysbiosis in periodontitis, we have used multispecies consortia and biofilm models to investigate the effects of saliva and serum (to model GCF) as nutrient sources on microbial composition and properties.

### Materials and Methods

Communities or biofilms composed of *Streptococcus dentisani*, *Streptococcus constellatus*, *Actinomyces naeslundii*, *Actinomyces odontolyticus*, *Parvimonas micra*, *Fusobacterium nucleatum*, *Veillonella parvula* and *Porphyromonas gingivalis*, were grown anaerobically at 37°C for 48 hours in unsupplemented nutrient medium (control) or medium containing either 20% saliva or 20% serum. Growth was monitored as OD<sub>600nm</sub> and composition determined by culturing on brucella agar. General proteolytic and gingipain activities were measured spectrophotometrically using fluorescent substrates.

### Results

After 48 hours, a small increase in growth was seen in saliva-supplemented medium compared to control, while 20% serum gave a 3-fold increase in community growth compared to saliva. In both communities and biofilms, saliva promoted growth of *S. dentisani* and *Actinomyces* species whereas serum promoted growth of anaerobic species such as *P. gingivalis*, *P. micra* and *F. nucleatum*. General proteolytic activity in consortia grown in serum-supplemented media was 5-fold higher than those in saliva-supplemented media. High levels of gingipain activity were observed, correlating with strong growth of *P. gingivalis*.

### Conclusions

These results suggest that saliva facilitates growth of health-associated communities and biofilms, enriched in streptococci and *Actinomyces* with low levels of proteolytic activity whereas serum promotes growth of proteolytically active, anaerobic bacteria associated with periodontal disease.

### Keywords:

Multispecies consortium, dysbiosis,, proteolytic activity

## Influence of Harmful Algal Blooms on the Biofilm Dynamics of a Full-Scale Biological Filtration System for Potable Water Production

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### **Background:**

Cyanobacterial harmful algal blooms (CHABs) present global challenges, raising public concern about safe drinking water production. Many drinking water treatment plants (DWTPs) face significant challenges in addressing CHAB-associated problems, such as cyanotoxins and taste- and odor-causing compounds. Recently, biological filtration systems (BFS) with granular activated carbons have received much attention for their ability to remove various emerging contaminants, including algal metabolites. For the successful operation of BFS, understanding the development and activity of biofilms in BFS is crucial. However, the underlying mechanisms (succession, community assembly, and functional profile) of biofilm development in BFS, especially under the impact of CHABs in source water, are not well understood yet.

### **Methods:**

Both water and biofilm samples were collected from a full-scale BFS in a DWTP that draws source water from Lake Erie. Field samplings were conducted during the typical CHAB season for two consecutive years. For the collected biofilm samples, biofilm quantity was monitored using the ATP biofilm kit. DNA was extracted and sequenced using Illumina MiSeq. Obtained sequences were processed using bioinformatics tools to monitor biofilm dynamics, relative abundance of functional metabolic pathways, and ecological networks in response to CHAB in the BAF.

### **Results**

Proteobacteria were dominant during CHAB seasons. Alpha diversity indices decreased during the severe CHAB but were relatively stable for the mild CHAB. When the functional pathways of biofilms were examined, higher gene abundance involved in lipid and amino acids metabolism was observed. The obtained results indicate that severe HABs significantly decrease bacterial diversity in BFS with the prevalence of CHAB-associated bacteria, while the function of biofilms in BFS showed increased metabolisms associated with algal organic matter utilization. Stochastic processes played a more important role in driving the community assembly in BFS, and the relative contribution of stochastic processes significantly increased during the severe HAB, leading to the development of less complex ecological networks.

### **Conclusions:**

This study examined the impact of CHABs on the development and dynamics of biofilms in BFS. Overall, the outcome of the study provides deeper insights into the community structures, functions, and assembly processes of biofilms in response to CHABs.

### **Keywords:**

biological filtration, algal blooms, biofilms

## Poster Line Up, Friday 28 June 13:30-15:00

### IMAGING AND/OR NOVEL TECHNIQS IN BIOFILM RESEARCH?

PG2-01

#### A Combination of Surface Science Methods in Biofilm Research

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##### **Introduction**

Biofilm research is a fascinating research field for many scientists. Old and novel techniques help to understand biofilm formation. The influence of the substrate on the bacteria and biofilm is of particular interest, for example, in biofilm reactors or bacteria tissue/implant interaction.

##### **Hypothesis and aims**

We aim to understand the early stages of biofilm formation (with and without the influence of shear) by addressing different methods to investigate the bacteria substrate interaction and biofilm composition. Further, we measure the spring constant of the bacteria as an indication of the viability of the cells.

##### **Methodology**

Scanning, fluidic, and lateral force microscopy (SFM, FluidFM, LFM) offer an impressive number of possibilities for biofilm investigations, such as imaging, determination of adhesion and lateral detachment forces, and of nanomechanical and electrical properties. Supplemented by the sulfuric acid ultraviolet absorption method (SA-UV-method) and other surface science methods (contact angle, zeta potential), a detailed image of the bacterial attachment/detachment on/off a substrate, the biofilm composition, and the major influencing parameters are obtained.

##### **Results**

By addressing different magnitudes of shear forces with the lateral force microscope, we determine the minimal detachment forces of single bacteria in the nN range. A correlation between the applied force and the number of moved bacteria, as well as between the number of detached bacteria and the substrate's surface energy, is obtained. In addition, scanning and fluidic force microscopy quantify the vertical adhesion forces under flow conditions and compare them to the absence of any flow. The SA-UV method reveals the composition of the conditioning film into proteins and polysaccharides.

##### **Conclusion**

Bacterial attachment/detachment highly depends on the substrate's surface energy. The surrounding pH, salinity, and surface topography can further steer them. However, the turgor pressure of the bacteria and, thus, their elasticity and size depend only on the salinity and pH but not on the surface material.

##### **Keywords:**

SFS, LFM, FluidFM, bacterial-adhesion, lateral-detachment-force

## USING LIGHT SHEET FLUORESCENCE MICROSCOPY TO IMAGE BACTERIAL BIOFILM DEVELOPMENT

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<sup>1</sup>*Ceitec MU, Brno, Czech Republic*

### **Background incl. aims**

Bacterial biofilms are a significant global health problem due to their resistance to immune system, antibiotics and the resulting persistence of infections. *Staphylococcus aureus* and *Pseudomonas aeruginosa* are the two most common microorganisms found in cystic fibrosis lungs and their co-infection is associated with poor clinical outcomes. To understand the multi-step process of biofilm formation we developed a new imaging method that enables continuous imaging of biofilm development.

### **Methods**

We used light-sheet fluorescence microscope (LSFM) with an integrated microfluidic system to monitor biofilm growth in situ. To visualize the biofilm-forming cells, we modified *S.aureus* and *P.aeruginosa* to stably express fluorescent proteins. The main components of biofilm matrix, such as extracellular DNA and polysaccharide intercellular adhesins, were labelled by specific fluorescent dyes.

### **Results**

LSFM enabled us to detect the distinct stages of biofilm formation in single species as well as in mixed biofilms. In our conditions *S.aureus* generates robust biofilm, in contrast, *P.aeruginosa* biofilm has the shape of a thin layer with dispersed clusters of cells. *S.aureus* with *P.aeruginosa* can coexist and form two species biofilm which is gradually dominated by *P.aeruginosa*. LSFM image stacks recorded from different orientations were used for 3D reconstruction, enabling the identification of the distribution of biofilm matrix components in single-species biofilms, along with the spatial arrangement of *S.aureus* and *P.aeruginosa* in the dual-species biofilm. We also tested the presence of competitive interactions and adaptive processes between *S.aureus* and *P.aeruginosa*. This was achieved by introducing conditioned media from co-culture cultivation into *S.aureus* biofilm, leading to prompt biofilm dispersion.

### **Conclusions**

LSFM with integrated microfluidic system represents a new tool for visualizing single species or mixed species biofilm formation. In addition, this system enables delivery of various agents into the mature biofilm to study their effect on bacteria by time-lapse imaging.

### **Keywords:**

bacterial biofilm, fluorescence microscopy

## The combined monitoring of oxygen and pH at the dental biofilm base under flow conditions

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Oxygen and pH are important factors that influence the metabolism and virulence of bacterial biofilms. In dental biofilms, low pH is responsible for the formation of caries lesions and has been studied extensively. In contrast, little is known about the oxygen saturation in dental biofilms and its importance for virulence.

Here we developed a method that combines confocal microscopy-based pH ratiometry with optode-based oxygen measurements. pH was measured inside the biofilm using the ratiometric dye C-SNARF-4, and oxygen was measured concomitantly at the biofilm base using a planar optode (oxygen-sensitive fluorophore: 2,3,4,5,6-pentafluorophenyl-porphyrin (PtTFPP), reference fluorophore: Macrolex Yellow).

As a proof-of-concept, we monitored the pH and oxygen saturation in 24-h *Streptococcus mutans* (DMS2523) biofilms grown under aerobic conditions in the presence of sucrose. Measurements were performed in 6 fields of view (FOVs) under static and different flow conditions (unstimulated flow: 0.8 mm/min, stimulated flow: 8.0 mm/min), with physiological saline (pH 7) containing 4 % sucrose as the flow medium. Experiments were performed in biological triplicates.

Average pH ( $\pm$ SD) in the biofilms fell to 5.59 ( $\pm$ 0.01) after 15 min of static conditions and remained similar, even after the onset of unstimulated ( $5.87\pm 0.05$ ) or stimulated flow ( $5.61\pm 0.04$ ), with little variation between individual FOVs (variance: static =  $0.01\pm 0.10$  SD, unstimulated flow =  $0.11\pm 0.34$  SD, stimulated flow =  $0.01\pm 0.12$  SD). In contrast, the oxygen concentration (% air saturation) varied greatly between different FOVs within the same biofilm under both static (maximal range: 9.8%-81.0%) and dynamic conditions (maximal range: 6.3%-81.2%). Interestingly, the variance between FOVs was significantly higher under static than under flow conditions (static:  $713.6\pm 28.7$  SD, unstimulated flow:  $421.5\pm 19.0$  SD, stimulated flow:  $416.9\pm 19.4$  SD).

### Conclusion

Combined pH ratiometry and optode-based oxygen measurements allow studying the interplay of two key factors for biofilm metabolism. Initial results from a simplified, highly acidogenic biofilm model system, suggest that the development of low pH is not dependent on anoxic conditions at the biofilm base. Further studies are warranted to investigate the relationship between pH and oxygen saturation in dental biofilms.

### Keywords:

pH-ratiometry, Oxygen sensors, Flow, Confocal-microscopy

## Simultaneous detection of joint and bone infections and antimicrobial susceptibility testing using isothermal microcalorimetry

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### Background

Accurate diagnosis of bone and joint infections can be challenging in cases of low-grade infections. 1-2 % of surgeries lead to hard-to-treat infections and thus, timely and accurate treatment is crucial for patient outcomes. The conventional gold standard of culturing from biopsies is time-consuming and labor-intensive, often leading to empirical antibiotic administration. Additionally, approximately 20% of cases yield false-negative results. This study aims to develop a rapid detection method (< 24 hours) of infections with low bacterial loads with simultaneous antimicrobial susceptibility testing (AST) in a single assay.

### Methods

We will measure metabolic heat signals from bacteria using isothermal microcalorimetry (IMC) that can detect as few as 1 CFU per sample. Surgical site tissue samples from an osteomyelitis pig model and patients will be exposed to a panel of carefully selected antimicrobials and a decision tree will be constructed for categorizing infecting pathogens by gram type and antibiotic susceptibility, distinguishing between coagulase-negative Staphylococci, *Staphylococcus aureus* (including differentiation of MRSA and MSSA), *Enterococcus* spp., and *Streptococcus* spp. as well as other clinically relevant species.

### Results

Our preliminary data indicate the feasibility of differentiating bacteria based on their sensitivity to selected antibiotics. *Pseudomonas aeruginosa*, *S. aureus*, *Proteus vulgaris*, and *Streptococcus suis* were successfully detected in tissue biopsies from the porcine osteomyelitis model in less than 5 hours, some even at counts as low as 100 CFU by using IMC. In a few cases, polymicrobial infection with *S. aureus* and *S. porcinus* could be detected within 24 hours, and in 1 biopsy from the porcine model, a polymicrobial infection harboring *S. aureus* and *P. aeruginosa* was detected in less than 10 hours using IMC. Ongoing method development aims to optimize the setup for biopsy material and deconvolution of signals from polymicrobial infections.

### Conclusions

The method has already demonstrated high sensitivity in directly detecting bacteria in biopsies, offering the potential to reduce false negative results seen from traditional culturing. With the use of carefully selected antibiotics, this approach could facilitate faster case-by-case treatment decisions, contributing to the era of personalized medicine.

### Keywords:

Osteomyelitis, diagnostics, detection, AST, IMC

## Biofilm matrix proteome profiling of *Stenotrophomonas maltophilia* wild type and its functional amyloid fiber deficient mutant

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### Background

Biofilms are communities of bacteria enclosed in a self-produced matrix. This matrix is a major contributor to biofilm-associated emergent properties, which cause the biofilm lifestyle to differ greatly from the planktonic lifestyle. Its composition varies between biofilms but commonly consists of exopolysaccharides, nucleic acids, lipids, and proteins. A particular group of proteins includes functional amyloid fibers (faps), commonly present in the matrices. Faps are well-described structural components and were associated with aggregation and enhanced biofilm formation but also with preventing loss of secreted compounds. However, the influence of lacking specific matrix components on the overall matrix composition remains unclear. We hypothesize that faps impact matrix proteomes, resulting in observable distinctions between producers and non-producers, driven by altered protein production, abundance, or retention.

### Methods

In this project, we used an environmental *Stenotrophomonas maltophilia* strain, harboring a fap operon and its mutant derivative ( $\Delta$ fap), incapable of producing fap proteins. Both strains were monocultured in flow cells under laminar, low-shear stress flow conditions ( $Re = 5.83$ ,  $\tau_{wall} = 3.65 \times 10^{-4}$  Pa) for 24 and 48 hours. Biofilms were examined by confocal laser scanning microscopy, and cells were quantified by CFU. The matrix proteins extracted from biofilms will be investigated using LC-MS/MS-based shotgun proteomics.

### Results

Examinations of the biofilms revealed aggregates of hexagonal patterns in both strains at 24 h. These distinctive patterns persisted even after 48 h in the  $\Delta$ fap mutant. In contrast, the wild type exhibited a denser and more uniform biofilm without such initial patterns. However, no significant changes in biofilm biovolumes were detected at any time. Notably, biofilms of the  $\Delta$ fap mutant had a significant 3.6-times higher cell number than wild-type biofilms after 48 h.

### Conclusion

These observations highlight the influence of faps on biofilm structure but also the burden of expressing the fap operon by impacting growth under these conditions. However, to continue proving our hypothesis, we will perform proteomic analyses to compare the matrix proteomes and evaluate whether distinctions in proteome composition are detectable beyond the expected absence of amyloid fibers in the  $\Delta$ fap mutant.

This study is funded by the European Research Council, grant ID: 101002208.

### Keywords:

biofilm matrix microfluidics amyloids proteomics

## Biofilm viability assessment with Calcein-AM and TMA-DPH assay in comparison to SYTO9 and PI staining

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### Background

Accurate assessment of bacterial cell viability within biofilms is crucial for evaluating the efficacy of anti-biofilm agents. A commonly used staining approach for this evaluation involves the pairing of DNA-binding fluorophores, SYTO9 and propidium iodide (SYTO9/PI). However, the application of these probes often leads to an underestimation of biofilm cell viability, attributed to the presence of extracellular DNA within the biofilm, and introduces other notable limitations.

This study aims to explore whether the staining method employing fluorophores, Calcein acetyl methyl ester (Calcein-AM), and 1-(4-Trimethylammoniumphenyl)-6-Phenyl-1,3,5-Hexatriene p-Toluenesulfonate (TMA-DPH), provides a more accurate reflection of biofilm viability compared to SYTO9/PI staining. Calcein-AM is selected for its specificity in staining metabolically active cells, providing a direct indication of viable cells within a biofilm. Meanwhile, TMA-DPH penetrates all cells and also provides insights into membrane fluidity patterns.

### Methods

Biofilms of Gram-positive and Gram-negative bacterial species, treated with antibiotics, were stained with combinations of Calcein-AM and TMA-DPH, as well as SYTO9 and PI, and were captured using Confocal laser scanning microscopy (CLSM). Subsequently, colony forming units (CFU) determination, considered the gold standard in microbiological viability assessments, was carried out on the same biofilms. Following this, CLSM images were analyzed using an ImageJ-based biofilm analysis tool that counts pixels from different fluorescent channels, and the data were compared to the corresponding CFU numbers of the treated biofilms.

### Results

A qualitative examination of CLSM micrographs stained with Calcein-AM and TMA-DPH revealed divergent patterns compared to images stained with SYTO9/PI in all bacterial species. Quantitative analysis in the Gram-positive species showed a positive correlation between the image analysis data of Calcein-AM and CFU, whereas no correlation was observed between SYTO9/PI staining and corresponding CFU counts. Work on the Gram-negative bacteria is currently underway.

### Conclusion

This study underscores the importance of accurate biofilm viability assessment for evaluating the efficacy of anti-biofilm agents. The preliminary results indicate that the utilization of Calcein-AM and TMA-DPH staining might provide a more precise method for assessing biofilm viability, emphasizing the superior efficacy of physiological state probes in contrast to DNA-binding fluorophores.

### Keywords:

Biofilm, CLSM, Calcein-AM, TMA-DPH, SYTO9/PI

## RESISTANCE MECHANISMS OF BIOFILMS

PG2-07

### Therapeutic strategies based on antagonistic resistance mechanisms between novel $\beta$ -lactam and carbapenem antibiotics to prevent resistance development on XDR *Pseudomonas aeruginosa* biofilms

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#### Introduction

Chronic biofilm-related infections by *Pseudomonas aeruginosa* (Pa) are difficult to eradicate. Novel  $\beta$ -lactam- $\beta$ -lactamase inhibitor combinations, such as ceftolozane/tazobactam (TL/TZ) seemed a promising option; however, it is not exempt from mutational resistance development. On the positive side, resistance to TL/TZ, mediated by specific mutations in the  $\Omega$ -loop of AmpC entail collateral susceptibility to carbapenems. Based on this antagonistic mechanism, treatment with the alternation of TL/TZ, followed by IM could avoid the development and/or selection of TL/TZ resistant mutants.

#### Methods

Reference strain PAO1 and a pair of isogenic XDR clinical strains (104-B7 and 104-I9, sensitive/resistant to TL/TZ, respectively) were studied. Experiments of single strains and mixed (104-B7 and 104-I9, 1:0.01 ratio) biofilms were carried out. Biofilms were grown at 30°C using the flow cell system. 48 hours-old biofilms were challenged during 6 days with either TL/TZ, 4/4 ( $\mu\text{g}/\text{mL}$ ) or the 2 days- alternation TL/TZ, IM 2 ( $\mu\text{g}/\text{mL}$ ) and TL/TZ. At the end of the treatments, biofilms were plated in Mueller-Hinton agar (MHA) and MHA with TL/TZ, to determine viable cells and resistant mutants, respectively. TL/TZ resistant mutants were characterized by susceptibility profiles and whole genome sequencing. Structural dynamics were monitored by CLSM and processed with IMARIS and COMSTAT programs.

#### Results

TL/TZ was not able to reduce biofilms counts of 104-B7 and led to the selection and amplification of resistant mutants (3 log approx. at day 6). Alternation with IM was able to eradicate TL/TZ resistant mutants. These results were also evidenced in CLSM structural dynamics analysis. Similarly, treatment with TL/TZ amplified the resistant strain 104:I9 in mixed biofilms (100% of the final population), while the alternation regimen reduced it to 2.33%. The resistant isolates showed mutations in AmpC associated with TL/TZ resistance and carbapenem collateral susceptibility: T96I (n=2), L244R (n=6) and a deletion aa236 $\Delta$ 7 (n=1).

#### Conclusion

The alternation strategy based on antagonistic resistance mechanism effectively avoids the selection and amplification of TL/TZ resistant mutants during treatment representing a potential therapeutic alternative against Pa chronic infections.

#### Keywords:

*Pseudomonas aeruginosa*, resistance, antagonistic, prevent

## Changes in capsule production and adhesion structures affect antibiotic susceptibility of *Klebsiella pneumoniae* biofilms

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### Background

Biofilms protect bacteria from antibiotic exposure and the host immune system, resulting in more complicated infections, need for longer treatments and increased risk of treatment failure. In this project, we study *Klebsiella pneumoniae*, a nosocomial pathogen that poses an increasing threat due to biofilm formation and multiresistance development. Recent studies in our lab show that there is a rapid selection of strains with increased biofilm capacity during infection, creating subpopulations with different virulence and potentially different antibiotic susceptibility.

We aimed to study how increased biofilm capacity, conferred by mutations affecting fimbriae, capsule production and cyclic di-GMP (c-di-GMP) regulation, impacts susceptibility to clinically relevant antibiotics.

### Methods

Biofilm formation of evolved mutants from two *K. pneumoniae* clinical isolates, C3091 and IA565, was assessed in the presence of nine antibiotics at concentrations ranging from 1/8 to 8 times the Minimum Inhibitory Concentration (MIC). After exposure, biofilm biomass and viability were evaluated by crystal violet staining and colony forming units determination, respectively. Viability was also evaluated following treatment of disrupted biofilms to investigate the protective effect of the biofilm structure.

### Results

Overall, mature biofilms were fairly tolerant to the majority of antibiotics tested. Changes in c-di-GMP regulation and fimbrial adhesins seemed to result in increased antibiotic tolerance, even in newly formed biofilms, while capsule overproduction did not seem to contribute to biofilm survival during treatment. Additionally, most antibiotics appeared to induce biomass production at sub-MIC concentrations at a strain and antibiotic specific manner. Furthermore, enhanced antibiotic tolerance might be attributed to subpopulations with slow growth rates or presence of an extracellular matrix, although such effects were only observed in response to specific antibiotics.

### Conclusions

Emergence of populations with increased biofilm capacity during infection could result in decreased susceptibility to antibiotic treatment, once again forewarning treatment challenges as the major burden of biofilms from a clinical perspective.

### Keywords:

Biofilms, *Klebsiella pneumoniae*, antibiotic tolerance

## Biofilm formation of carbapenemase-producing bacteria isolated from hospital surfaces in low- and middle-income countries

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### Background

Carbapenemase-carrying sequence type 15 (ST15) *Klebsiella pneumoniae* (*bla*<sub>NDM</sub> and *bla*<sub>OXA-48</sub>-like) was recovered across neonatal ward surfaces in Pakistan during the BARNARDS study (Burden of antibiotic resistance in neonates from developing societies) 2015-2018. Core genome SNP analysis showed isolates were identical to those causing neonatal sepsis. Biofilm production by carbapenemase-producing bacteria may inhibit effects of treatment/cleaning regimes in healthcare settings in low- and middle-income countries (LMICs), potentially contributing antimicrobial resistance (AMR) reservoirs on hospital surfaces to infections and outbreaks. Aim: This study evaluates *in vitro* biofilm formation, and resistance levels to antibiotics/biocides in the sessile state using a sub-collection of 77 BARNARDS isolates (mostly ST15 *K. pneumoniae* recovered from surface, rectal and blood samples).

Methods: Biofilm formation was quantified using the modified microtiter-plate test and validated by confocal and scanning electron microscopy. The minimum inhibitory (MIC), and the minimum biofilm inhibitory and eradication concentrations (MBIC and MBEC) of antibiotics and disinfectants were determined using broth microdilution. Whole genome sequencing was performed using Illumina MiSeq and Oxford Nanopore Technologies, to allow for *in silico* selection of genes involved in biofilm formation and tolerance for expression analysis; investigated by reverse transcriptase quantitative PCR (RT-qPCR).

### Results

The 77 isolates exhibited phenotypic resistance to ampicillin (100%), ceftazidime (97.4%) and gentamicin (84.4%), first-line therapy for neonatal infection recommended by the World Health Organisation. All 77 strains (100%) were resistant to ertapenem, and some resistance was observed against meropenem (66%) and imipenem (38%). All isolates were however susceptible to isopropanol (3.13 – 12.5%), sodium hypochlorite (0.5-1.02g/L), hydrogen peroxide (0.01171875- 0.0234375%), and ammonium chloride (5-10%). Strong ability to form biofilms was observed for n=8/77 ST15 *K. pneumoniae* strains, and for these eight, MBIC and MBEC of ampicillin and gentamicin were the same or higher (>32µg/mL) to their planktonic counterparts' MICs. MBIC and MBEC of biocides and biofilm gene expression results, to follow.

### Conclusions

This *in vitro* dataset emphasises the ability of isolates originating from LMIC wards to produce biofilms that may be residing on hospital surfaces. This dataset will provide evidence of whether commonly used biocides remain effective and will endorse tailored infection prevention and control practices.

### Keywords:

AMR, biofilm, *Klebsiella*, hospital, LMIC

## Horizontal Gene Transfer Amongst Microbial Communities On Plant-Based Food

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### Background

Horizontal gene transfer, through which bacteria acquire exogenous plasmids, plays a pivotal role in the proliferation of antibiotic resistance genes, intensifying the antibiotic resistance crisis. Investigating the capacity of microbial communities in direct contact with humans or animals, such as those on raw vegetables and plant-based foods, to accept and disseminate crucial plasmids is essential. This transfer is particularly concerning for food processing biosafety, as interspecies interactions may facilitate the spread of harmful genetic traits like resistance genes. This study evaluates the ability of microbial communities on vegetables to acquire plasmids and identifies the recipient species.

### Methods

This research examined the transfer of the IncP1 plasmid pB10 from *Pseudomonas putida* KT2440 and *Escherichia coli* Nissle 1917 to the native microbial flora of various unprocessed vegetables, including spinach, potatoes, carrots, bean sprouts, tomatoes, and arugula. A dual fluorescence gene reporter system was employed to detect transconjugants, distinguishing donors by red fluorescence (mCherry) and transconjugants by green fluorescence (GFP). The transconjugants were subsequently isolated, sorted, and identified at the species level through 16s rRNA sequencing using Oxford Nanopore Technology.

### Results

The presence of transconjugants in all tested vegetables confirms the occurrence of plasmid transfer within these microbial communities. The varying frequencies of transconjugants across spinach, potatoes, carrots, bean sprouts, tomatoes, and arugula highlight the differential susceptibility of these communities to plasmid acquisition. Preliminary analysis of 16s rRNA sequencing has begun to shed light on the species composition of these transconjugant communities. Concurrently, a plasmid-focused metagenomic analysis is being conducted to elucidate the diversity and prevalence of native plasmids within these microbial ecosystems.

### Conclusion

The capacity of microbial communities on raw vegetables to uptake the IncP1 plasmid is evident, with ongoing identification of recipient species. This knowledge is crucial for selecting specific bacterial strains as biological control agents, thereby enhancing biosafety in food production and reducing the risk of plasmid transfer in our food supply.

### Keywords:

HGT Antibiotics Resistance Plasmid Food

## Collective antibiotic-mediated interactions in a dense microbial population expressing efflux pumps

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The emergence of antibiotic resistance is a significant threat today. It has been pointed out that antibiotic resistance can arise from collective phenomena between cells, where the density of the population – that in biofilms is higher than in planktonic cultures - plays a relevant role. A common way bacteria become resistant is through multidrug efflux pumps, which actively pump antibiotics outside of the cell. These pumps have been shown to be more expressed in biofilms. We suggest that the expression of efflux pumps can lead to antibiotic-mediated interactions that are influenced by the population density.

We develop a minimal mathematical model to study how a cell population's ability to keep low internal antibiotic levels can change. This depends not only on cells' pumping efficiency but also on population density and composition.

Cells interact weakly when their density is low, since they don't perturb significantly the common environment. On the contrary, as we show, interactions emerge at higher densities leading to competition effects between cells overexpressing efflux pumps. Due to this, the efficiency required for pumping to sustain a specific internal antibiotic depletion rises in a non-linear fashion with the population density. On the other hand, cells that behave mainly as antibiotic buffers show a cooperative behaviour. These results gain significance in heterogeneous populations where cells have varying pumping efficiencies. This offers a framework to evaluate the optimality of different adaptation strategies in relation to the ability to maintain low levels of internal antibiotic.

### **Keywords:**

efflux-pumps, antibiotic resistance, mathematical modelling

## Identification of genes involved in aggregate associated antimicrobial tolerance of *Pseudomonas aeruginosa* using Tn-Seq guided discovery

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It is estimated that about 60-70% of hospital acquired infections are caused by biofilms. Since current treatment regimens in many cases fail to cure biofilm-based infections, it is critical to identify the mechanisms which underlie biofilm-based antimicrobial tolerance.

In biofilms, the bacteria grow in densely packed aggregates concealed in a self-produced matrix of exopolymers. In this life-mode, the bacteria can tolerate high doses of antibiotics.

We use a super-saturated *P. aeruginosa* PA14 transposon (Tn) mutant library consisting of more than 800.000 unique mutants, each with a single transposon inserted at a random position in the genome, to identify mutants with increased susceptibility towards a panel of antibiotics.

The transposon mutants are grown in agar plates under conditions where each mutant forms an agar-embedded aggregate.

In this aggregate model, the bacteria start to significantly reduce their growth rate after 16-18 hours. Though antimicrobial susceptibility increases with aggregate age even relatively young aggregates display tolerance which is increased up to 10-fold compared to the planktonic MIC value.

Furthermore, the level of c-di-GMP – as measured through a c-di-GMP-monitor - is significantly higher in the aggregates compared to planktonic growth, which confirms that the aggregates represent a biofilm-like growth physiology.

For a single experiment, we use several large agar plates that combined harbor more than 150.000 aggregates. The aggregates are treated with antibiotics, washed, and then disintegrated. The identity and relative number of the mutants present in the pool of antibiotic-treated versus the non-treated bacteria are then determined by Tn-Seq analysis. Under the right selection conditions, Tn-mutants with higher susceptibility will be selectively depleted from the antibiotic-treated pool. Subsequently, all the genes are ordered according to their potential importance for biofilm-associated antibiotic tolerance. The identified genes serve as the starting point for exploring the proteins and mechanisms that are involved in biofilm-associated antibiotic tolerance.

### **Keywords:**

Antimicrobial tolerance, Tn-Seq, Aggregate biofilms

## Biofilm Formation Dynamics and Antimicrobial Resistance in Coagulase Negative Staphylococci: Implications for Clinical Management

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### Background

Although ignored for many years, Coagulase Negative Staphylococci (CoNS) have an important role in causing hospital-acquired infections and their incidence has been increasing in recent decades in parallel with medical advances. The main aims of this study were to compare the biofilm-forming capacity of CoNS isolated from humans with infections and from clinical specimens contaminated with this bacterium, to study the association between biofilm formation and antimicrobial resistance and to evaluate the effect of tetracycline in reducing the biofilm mass formed by CoNS.

### Methods

The 119 isolated strains were assessed for their ability to form biofilm using the microtiter biofilm assay. Statistical analysis was used to find associations between biofilm formation capacity and antimicrobial resistance, distinct species and type of isolate. The antimicrobial susceptibility of the 4-hour-old biofilms was evaluated in relation to the minimum inhibitory concentrations (MIC) and, 5x MIC and 10x MIC of tetracycline, and the biomass reduction was measured.

### Results

All isolates had the ability to form biofilms. There were no statistically significant differences between resistant and susceptible CoNS for any of the antibiotics analyzed (oxacillin, trimethoprim-sulfamethoxazole, tetracycline and gentamicin) or between *S. epidermidis* and other species, i.e. none of these parameters influence biofilm formation. However, significant differences were found between CoNS causing infections and CoNS contaminating clinical specimens, with the former being more prone to form biofilm. Tetracycline was able to reduce the biofilm mass but was unable to eradicate the biofilm at the maximum concentration used.

### Conclusions

Our results provide important information on the biofilm-forming capacity of CoNS and may have implications for the development of new therapeutic strategies aimed at reducing biofilm formation and consequently the number of infections in hospital environments.

### Keywords:

Biofilm, CoNS, Antimicrobial Resistance

## Influence of antimicrobial resistance in bacteria of veterinary and public health importance on surface biofilm formation

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### **Background (including aims)**

The persistence of pathogens which harbour resistance to antimicrobials is of growing concern. Despite efforts to reduce antimicrobial usage, antibiotic-resistant pathogens continue to persist in the environment and disseminate within and between livestock premises. Biofilm formation facilitates bacterial survival and persistence in farm environments. This study aimed to assess the biofilm-forming ability of antibiotic-resistant bacteria of veterinary and public health importance, such as livestock-associated methicillin-resistant *Staphylococcus aureus*, multi-drug resistant (MDR) *Escherichia coli* and MDR *Enterococcus* spp. including vancomycin-resistant *E. faecium*. Comparisons were drawn between these antibiotic-resistant pathogens and their antibiotic-sensitive counterparts, to investigate the relationship between antimicrobial resistance and biofilm formation on surfaces commonly found in farm environments.

### **Methods**

A panel of antimicrobial resistant and sensitive isolates was obtained from the APHA culture collection of UK isolates. Biofilm-forming ability was initially assessed on polystyrene using the crystal violet assay (CVA), at a range of temperatures (4°C, 10°C, 20°C, and 37°C). Results from the CVA informed the selection of a smaller panel of isolates which were assessed for biofilm formation in short-term realistic models. These models assessed biofilm formation on a range of commonly used surface materials found in farm environments (concrete, poplar wood, brick, PVC, and stainless steel), following 48-hours incubation.

### **Results**

A wide range of biofilm-forming abilities were observed amongst the isolates, from non-biofilm formers to strong biofilm formers. Differences in biofilm-forming ability were observed in some cases between the antibiotic-resistant and antibiotic-sensitive isolates. Degree of biofilm formation was influenced by incubation temperature as well as surface material. Greater biofilm formation was observed on porous surfaces (concrete, brick, and poplar wood) than on non-porous surfaces (PVC and stainless steel).

### **Conclusions**

Biofilm-forming ability varied between bacterial species and irrespective of AMR profile. Biofilm formation is a complex process; regulated by intrinsic and extrinsic factors, including temperature and surface material. Further study should be undertaken to understand the relationship between antibiotic resistance and biofilm formation, as well as biocide tolerance, and the transfer of AMR genes within biofilms. Additional studies of longer duration would also prove beneficial in understanding biofilm development in farm environments.

### **Keywords:**

Surface, Veterinary, One-Health, AMR, Temperature

## Experimental ex vivo analysis of microbial population dynamics reveals that antifungal agents negatively drive bacterial microbiome dynamics with and without *Candida albicans*

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### Background

*Candida albicans* is a prolific opportunistic pathogen and resides within the mucosa-associated microbiome in the human body. Oral antifungal agents are frequently used to control superficial mycoses and for prophylactic use, yet we have little understanding how these can influence complex microbial communities. This study aimed to test how the application of antifungals affected a complex microbiome.

### Methods

Saliva was pooled from 12 individuals and used as starter biofilm cultures for ex-vivo experiments (n=3). Complex bacterial biofilms ± *Candida albicans* were formed over 5 days ± fluconazole (FLU) or amphotericin B (AMB), with biofilms sampled every 24 h for 120 hrs. DNA was extracted with propidium monoazide to amplify live cell DNA. Microbiome analysis was performed using long read Nanopore sequencing, supplemented with qPCR for bacterial (16S) and fungal (ITS) quantification.

### Results

Data showed that biofilms took 5 days to reach maturity. Antifungal treatments in complex communities without *C. albicans* revealed significant changes in microbial composition, particularly FLU driving *Staphylococcus aureus* overgrowth. When *C. albicans* was present, the microbiomes were more relatively stable and tolerant to antifungal treatment, though the phenotype (e.g. yeast or hyphal) was shown to influence community structure.

### Conclusions

It is often assumed that antifungal agents have no impact on bacterial communities, yet we have shown that antifungals cause biofilm dysbiosis. We also show that when *C. albicans* resides in complex microbiomes, that they are tolerant to antifungal therapy. Together these data have implications for antimicrobial stewardship due to collateral damage to bacterial populations from antifungal therapy.

### Keywords:

AMR, AFR, Microbiome, *Candida*, Polymicrobial

## SPATIAL BIOFILM ORGANIZATION

PG2-16

### Cell aspect ratio is a mechanical winning strategy in microbial competition

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Bacteria's competition for nutrients and territory drives biofilm evolution. The factors determining the outcome of competition among diverse bacterial species have a broad impact on a wide range of pathological, environmental, and microbiome interactions. While motility-related traits and specific molecular mechanisms have been identified as potential winning attributes in bacteria, a shared and universally conserved feature determining competition remains elusive. Here, we demonstrate that a simple morphological feature of individual bacteria, cell aspect ratio, provides a winning trait for the population. Using range expansion experiments, we show that relatively longer bacteria robustly conquer the expanding front, even when initially in minority. Using an agent-based model of dividing bacteria, we reveal that the takeover mechanism is their emergent collective alignment: groups of locally aligned bacteria form "nematic arms" bridging the central region of the colony to the expanding front. Once at the front, bacteria align parallel to it and block the access of shorter bacteria to nutrients and space. We confirm this observation with single-cell experiments and further generalise our findings by introducing a generic continuum model of alignment-dominated competition, explaining both experimental and cell-based model observations. Moreover, we extend our predictions to spherical range expansions and confirm the competitive advantage of being longer, even though the effect is less pronounced than in surface-attached colonies. Our results uncover a simple, yet hitherto overlooked, mechanical mechanism determining the outcome of bacterial competition, which is potentially ubiquitous among various bacteria. With the current advances in genetic engineering, varying aspect ratios can work as a simple tunable mechanism for the on-demand setting of the outcome of bacterial competitions with widespread implications for biofilm control.

#### **Keywords:**

morphology, *Escherichia coli*, range expansion

## Motility mediates satellite formation in confined biofilms

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Biofilms are commonly thought of as 2D habitats for microbial life, but there are niches where bacteria grow as 3D conformations. So, what are the mechanisms controlling bacterial growth in these environments? Especially, how does cell motility drive spatial organization?

We use confocal laser-scanning microscopy to image mono-clonal spherical colonies of the model organism *E. coli* K-12. We find satellite colonies emerge around *E. coli* colonies embedded in semi-soft agarose (0.3%). We identify the key structures controlling colony morphology, by studying mutants lacking surface structures involved in adhesion or motility processes and required to form biofilms. By linking the experimental observations with the results of classical motility assays, we conclude that the emergence of satellite colonies is a direct consequence of flagella-based motility. We also find that satellites appear in different nutritional media, but its manifestation is highly sensitive to changes in the extracellular matrix elasticity.

Complementing these experiments, we build a mathematical model of how cell motility drives the spatial organization of spherical colonies. When paralleled with computational simulations, our study suggests that satellite formation allows bacterial communities to spread faster, while retaining the close interactions and protection supplied by the community.

Overall, our research provides fundamental insights into how bacteria grow and invade three-dimensional settings and has potential implications to find strategies to combat bacterial spread.

### **Keywords:**

3D growth, motility, morphology, *E.coli*

## The Small basic protein (Sbp): structure and spatio-temporal patterning in *Staphylococcus epidermidis* biofilm formation

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### Introduction

*Staphylococcus epidermidis* is one of the most frequent nosocomial pathogens which cause implant-associated infections through biofilm formation. Its biofilm assembly relies on the production of an extracellular matrix, known as the matrixome. In effort to understand specific molecular interactions within the matrixome, a so far uncharacterized, 18 kDa protein was identified named small basic protein (Sbp).

### Goal

The aim is to characterize and to elucidate the temporal-spatial distribution of Sbp in *S. epidermidis* biofilms.

### Methods

To investigate the dynamic Sbp matrix three Sbp-isoforms were constructed in which Sbp is fused to an ALFA-tag located at different positions within the protein. The constructs were cloned in pRB473 (constitutive promotor) and pHB3 (xylose-inducible promotor) plasmids and were tested (growth curves, biofilm formation, WB, CLSM).

### Results

Bioinformatic analysis revealed a structured protein head with  $\beta$ -loops and  $\alpha$ -helices, along with an unstructured N-terminal tail. Analyzing the three cloned constructs via WB no degradation products were detected and no impact on the growth rate was identified. Using CLSM, a so far unique spatial distribution pattern of Sbp within matrices became apparent. Native Sbp accumulates at early timepoints at the interface between basal biofilm layers and the substratum (forming a carpet like structure). The same is true for sbp-ALFA at position 508 and 466. In mature biofilms Sbp is enriched in both biofilm matrix and at substratum with no significant differences between the strains. In higher parts of the biofilm Sbp partially co-localizes with ALFA independent of tag position whereas these carpet like structure of ALFA could only be detected in sbp-ALFA 508.

### Summery/Conclusion

All Sbp-ALFA fusion constructs are stable and reconstitute the wildtype biofilm phenotype in  $\Delta$ sbp strains. The Sbp-ALFA nt508 recapitulates wildtype Sbp distribution in biofilms the best, so this construct can be used for high resolution microscopy and to decipher specific Sbp- matrix interactions and their functional importance during *S. epidermidis* biofilm assembly. At present, it is unclear whether and how Sbp can be exchanged between *S. epidermidis* sbp-producing and non-producing cells. Co-culture experiments will facilitate to follow Sbp production, potential repositioning, fate and ex-change of Sbp between different subpopulations and strains.

### Keywords:

spatio-temporal patterning  
biofilm-matrix  
*Staphylococcus epidermidis*

## Yeast biofilms and colonies: Common features and differences between differentiated cell subpopulations

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### Background

Yeasts form different multicellular communities such as colonies (laboratory strains) or colony biofilms (wild strains) in which they use different strategies to survive unfavorable environmental conditions. Wild *Saccharomyces cerevisiae* strains form complex colony biofilms in which the cells differentiate into subpopulations that perform specific functions which provide protection to the whole structure. The surface cells have active transporters that expel harmful compounds, the central cells produce a protective extracellular matrix which fills the central cavity, protects the structure from desiccation and serves as a nutrient reservoir, and the lower cells form pseudohyphae that attach the structure to the substrate. When wild cells encounter favorable conditions with an abundant carbon source over a long period of time, they domesticate, switch off the biofilm properties and form smooth colonies consisting of two main cell subpopulations that develop cooperatively and ensure the longevity of the population. These are metabolically active U cells in the upper colony layers and starving L cells in the middle/lower colony part. An important question is how the differentiated cells of biofilms and colonies differ in their metabolism.

### Methods

We used microscopic, biochemical and OMICs approaches together with genetically modified strains to analyze differentiated cell subpopulations of differently structured yeast communities.

### Results and conclusions

Transcriptomic and proteomic analyzes enabled us to identify common features and differences between cell subpopulations of colony biofilms and smooth colonies. The main differences concern nitrogen/amino acid metabolism, respiration, defense against ROS and stress, and accumulation of storage compounds. Cells with some similar characteristics are present, but they form subpopulations that are localized at different sites in both structures. In addition to the specifically localized subpopulations that develop in a coordinated manner, cells that evade coordination and overgrow the rest of the population also develop in the structures. Proteomic analyzes revealed that these cells, which have non-functional Whi2p, have different properties than the rest of the population. Our results shed light on the importance of metabolism for the development of different types of multicellular structures.

The work was supported by GACR23-06368S and RVO 61388971.

### Keywords:

yeast, biofilm, colony, metabolism, differentiation

## Aggregates in action: Dynamics and spatial organization in biofilm formation

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### Background

Bacteria have been known to exhibit two primary lifestyles: either as free-floating planktonic individuals or as attached biofilms on surfaces. However, suspended bacterial aggregates are increasingly being emphasized as significant forms of bacterial communities distinct from surface-attached biofilms and are frequently found in clinical and environmental settings. Despite similarities, these non-attached cell clumps exhibit varying sizes and different formation mechanisms. In this study, our aim was to compare biofilm characteristics depending on whether they originated from either planktonic cells or bacterial aggregates. Our focus was on spatial organization, cell viability, and the interplay between growth rates, detachment processes, and consequential recolonization.

### Methods

To this end, photo-switchable proteins expressed on the surface of *Escherichia coli* facilitated aggregation and allowed for control of aggregate sizes. Aggregates were introduced under both static and flow conditions to monitor their fate as nuclei for biofilm development as well as their interaction with already established biofilms. Live/Dead staining with confocal laser scanning microscopy (CLSM) and advanced image processing were used to provide a comprehensive understanding of the dynamics of this biofilm system.

### Results

Changes were observed in the initial phase of biofilm formation, specifically concerning the attachment rates of planktonic cells, across varied environmental conditions. There was a lack of significant detachment of surface-attached planktonic cells at the initial stages of biofilm formation under varying flow conditions (e.g., flow rates and media composition). However, further experiments are ongoing in our lab to further substantiate these observations and to assess the biofilm structural characteristics under the different formation conditions over longer time periods.

### Conclusions

Characterizing and understanding the dynamics of biofilm-aggregate interactions will enable a better understanding of the biofilm life-cycle in order to develop new technologies for the exploitation of biofilms as well as new modalities for biofilm removal and control.

### Keywords:

Biofilm, Formation, Aggregates, CLSM

## Experimental methodology to assess bulk and flow effect on biofilm morphology and bacterial motility

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### **Background incl. aims.**

Biofilms growth in confined environment, such as conditions typical of space exploration capsules and stations, pose an issue on life support systems that sustain the ideal homeostasis for human life in space. Preventing those systems loss of functions due to biofouling is a pivotal achievement from exiting Earth orbit and take humans on deep space explorations. A recurring fouling-related issue was found in the water recycling plant on the International Space Station (ISS), where biofilm develops in-flow on a solid-liquid interface. This situation is further complicated by the poorly understating of microgravity effect on biofilm. The role of combined bulk and flow driven stresses on biofilm growth for this application is still under discussion. In our work we present a methodology to quantitatively analyse the synergistic effect of gravity and shear stress on bacterial motility and biofilm formation. The understanding of cell motility is a key aspect that affect the early stages of biofilm formation.

### **Methods**

*Pseudomonas fluorescens* SBW25 was used as biological system to test our experimental setup. A microfluidic setup based on direct visualization by CLSM and Time-lapse microscopy of a rectangular channel was employed. Bacterial trajectories and biofilm morphology were quantified by image analysis and further analysed by mathematical models based on Persistent Random Walk theory. The methodology of this study can be applied for both on Earth and Space related application.

### **Results**

Statistical analysis identified the minimal number of trajectories needed to obtain significant quantitative measurements of bacterial motility. Flow and gravity induced stress anisotropies show a measurable effect on bacterial motility and biofilm morphology. Transport efficiency and shear drag are identified as two competitive flow related mechanisms, that affect biofilm formation.

### **Conclusions**

The setup here proposed provides a proof of concept of the relevance of gravity on biofilm development, suggesting further future investigation to fully understand the complexity of this system in microgravity conditions.

### **Keywords:**

Motility, flow, biofilm morphology

## Antibiotic tolerance of biofilms emerging from multicellular effects of antibiotic efflux

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### **Background incl. aims**

Biofilms are multicellular assemblages of bacteria living in a self-produced extracellular matrix. One characteristic of biofilms is that they are difficult to kill. Different mechanisms, like the development of highly tolerant persister cells or increased expression of efflux pumps, which pump certain antimicrobials out of the cell, make them tolerant. Our central hypothesis is that efflux pump activity causes emergent antimicrobial tolerance of multicellular bacterial populations, through the interplay of efflux-mediated spatial interactions and efflux-linked persistence. Methods: To test the hypothesis, we are combining computational modelling with information gained from 3 types of multicellular assemblages, i.e. colonies on agar, multicellular populations grown in microfluidic devices, and 3D biofilms grown in flow chambers. We are currently generating strains that differ in their levels of efflux activity. The strains will be mixed and then cultivated together in the 3 model systems. In colonies, the link between structure and spatial patterns of gene expression will be characterized. Experiments in microfluidic devices will be used to determine the interactions range of cells with high efflux activity on neighboring susceptible cells. The results will be used to construct individual-based models that predict the effect of efflux on biofilm structure and antimicrobial tolerance. Flow chamber 3D biofilms will be generated to test the model predictions. The results will shed light on the interplay between spatial biofilm organization, multidrug efflux and antimicrobial tolerance.

### **Keywords:**

tolerance, persistence, spatial interactions, AcrAB-TolC

## Linking adenosine triphosphate (ATP) levels and single-cell transcriptomics in biofilms

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### Background

The spatial organization of biofilms generates gradients that lead to phenotypic heterogeneity. This creates metabolic variation in the community, where some cells are in a slow-growing or dormant state hypothesized to cause tolerance. To grasp the impact of the physio-chemical heterogeneity on gene expression diversity and tolerance we aim to link expression profile to spatial position and metabolic state. This insight can improve anti-biofilm strategies in clinical settings where tolerance and recalcitrant infections cause complications.

### Method

Adenosine triphosphate (ATP) is a marker of bacterial metabolic activity. Here, an ATP reporter, previously used to associate ATP and persisters, was inserted into the chromosomes of *Escherichia coli* AR3110, *Kluyvera cryocrescens* MB101 and *Pseudomonas aeruginosa* PA14. The fluorescent protein reporter is composed of a circularly permuted superfolder GFP and an ATP-binding subunit FOF1-ATP synthase, which reports the level of extracellular and cytosolic ATP. Using flow cytometry, we show that the reporter mirrored different growth stages of *E. coli* well. For *K. cryocrescens*, the signal was weak resulting in too low resolution. The reporter did not yield a signal that was detectable for *P. aeruginosa*. As the reporter worked well in *E. coli* biofilms were cultivated in a microfluidic channel system where ATP heterogeneity was imaged by Confocal Laser Scanning Microscopy (CLSM). Next, the active and inactive cells will be sorted into two subpopulations based on fluorescence intensity, and single-cell RNA sequencing will be performed. Sequencing results will enable the identification of significant, differential expression genes between active cells and inactive cells in biofilm.

### Results

The ATP reporter was successfully integrated in *E. coli*, resulting in varying fluorescence output as detected by microplate fluorometry, CLSM, and flow cytometry. Subpopulations with different fluorescence intensities were identified in planktonic bacteria over a 48 hour time period. The next steps are sorting the subpopulations in biofilms based on fluorescence followed by single-cell RNA sequencing, which are ongoing experiments.

### Keywords:

Biofilm heterogeneity;Metabolic activity;Single-cell transcriptomics

## Development of NAM-FISH for the study of biofilms formed in an advanced 3D substrate

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Cystic fibrosis (CF) is commonly associated with persistent polymicrobial biofilm infections that eventually lead to premature death. Therefore, it is crucial to develop new models/platforms and methods to study biofilms, to better understand them and to develop targeted strategies to prevent and treat biofilm-associated infections.

The main objective of this study was to implement and validate Nucleic acid mimics-Fluorescence in situ hybridization (NAM-FISH) on biofilms cultured in a novel 3D substrate – the Universal Bac3Gel - that reproduces key properties of the human mucus. Two pathogens relevant to CF, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*, were selected to generate single- and dual-species biofilms. Biofilms were evaluated by confocal laser scanning microscopy (CLSM) to determine the spatial distribution of *P. aeruginosa* and *K. pneumoniae* within the biofilm and to infer their interactions. Validation experiments showed that NAM-FISH detected  $\approx 84\%$  of the cells detected by 4',6-diamidino-2-phenylindole (DAPI), with a correlation coefficient of 0.97 between the two methods. The application of NAM-FISH to single-species biofilms showed that both species were able to form biofilms, reaching a total number of cells detected by this method of log 8.9 and log 8.6 cells/mL for *P. aeruginosa* and *K. pneumoniae*, respectively. Regarding the cultivability results, *P. aeruginosa* showed higher counts after 48h of culture, while *K. pneumoniae* showed higher counts after 24h. For dual-species biofilms, it was observed that both species were able to form biofilm and reach values of total cells (detected by NAM-FISH) similar to those obtained in single species, around log 8.6 and log 8.2 cells/mL, for *P. aeruginosa* and *K. pneumoniae*, respectively. In conclusion, the combination of NAM-FISH with CLSM showed that the species exhibited a co-aggregation distribution, meaning that this consortium could be beneficial for both species. Overall, it was clear that the application of NAM-FISH in biofilms formed in Universal-Bac3Gel has great potential for observing the 3D structure of the biofilm within the hydrogel.

### Keywords:

fluorescence in situ hybridization;Bac3Gel;biofilms;spatial organization.

## Bacterial communication in porous media flows

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Fluid flows are major features of many bacterial environments, and frequently affect the bacterial activity in unforeseen ways. Advective transport can wash away the chemical signals used by bacteria to communicate, thus disturbing processes such as quorum-sensing (QS) and creating heterogeneities in space. Since QS mediates the attachment and detachment of bacterial colonies, for example through regulating the production of surface adhesins or surfactant proteins in the case of *Staphylococcus aureus*. Thus, we hypothesized that flow plays a fundamental role in the attachment/detachment of colonies, not only through the effect of the hydrodynamic stress, but also by controlling the biological response. In this work, our goal is to understand how complex couplings between flow and the biological response controlled by QS shape biofilm clogging in porous structures, as found in e.g., the hyporheic compartment or in biofilters used for wastewater processing.

To study such systems, we used microfluidic approaches, in particular PDMS/glass chips, with a range of different designs of porous systems, from a single pore to T-junction and a honeycomb like porous network. We further combined this approach with fluorescence microscopy of a dual-labelled *Staphylococcus aureus* strain (mKate constitutive, GFP corresponding to QS activation). This approach allows us to simultaneously visualize the spatiotemporal dynamics of clogging, cells viability and QS.

We observed a rich spectrum of dynamics in the complex porous network, where clogging and communication phenomena alternate between pores through time. In order to understand the basis of these dynamics, we studied further a minimalist system consisting of a single T-junction, which also features spatiotemporal fluctuations. Revealing the fundamental mechanisms of QS in complex geometries such as porous media may provide important insight for the development of strategies for controlling beneficial or harmful bacterial behaviors in nature, in medicine and in industry.

### **Keywords:**

Porous media, Quorum-sensing, Transport phenomena

## Couplings between mass transport, growth and flow-induced removal control the spatio-temporal dynamics of *Pseudomonas aeruginosa* biofilms in microchannel flows

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Hydrodynamic conditions play a particularly important role on the different stages of biofilm development. Advection can efficiently transport solutes, including nutrients and biocides, but can also wash away molecular signals used in cell communications. Flow can trigger specific stress/strain detection systems, which in turn can affect the behavior of bacteria. Hydrodynamic stresses can further modify the matrix of extracellular polymeric substances, such as its composition, shape and density, modulate mechanical properties or induce detachment at later stages. We hypothesized that a better understanding of the complex couplings between mass transport, growth and flow-induced removal could open to way towards a better control of biofilms in bioengineering applications, such as bioremediation, wastewater processing or the production of value-added products. Our goal is therefore to better understand the fundamentals of these couplings.

Here, we study the development of *Pseudomonas aeruginosa* biofilms in microchannel flows at constant flow rate. We combine microfluidics with time-lapse fluorescence microscopy, image analysis, micro rheology, mathematical modeling and molecular biology.

In the early stages, when single attached cells are exposed to flow and small microcolonies start to develop, we show that hydrodynamic conditions drive cell orientation, growth speed, early microcolony formation and near surface motility. After several days, we find that biofilm development features complex spatio-temporal dynamics with strong couplings between growth, resources transport and detachment. In the late stages, in particular, we find the spontaneous occurrence of large temporal oscillations that stem from a competition between flow-induced sloughing and growth. We further study the different parameters controlling detachment and propose a novel modeling approach to such problems.

### **Keywords:**

Microfluidics, biophysics, modeling, transport *P.aeruginosa*

## Regulation of yeast lifestyle in spatially structured, differentiated communities

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### Background

On solid/semi-solid surfaces, yeasts, like other microbes, form spatially structured communities - colonies and various types of biofilms. The cells that form these structures interact and differentiate into precisely localized cell subpopulations that perform different functions, often for the benefit of the entire community. The types of differentiated cells and their position in the structure vary considerably in biofilms and colonies. For example, domesticated strains of *Saccharomyces cerevisiae* adapted to abundant nutrients form colonies on the agar in which vital cells in the upper and stressed cells in the lower regions develop in a coordinated manner, whereas wild strains of *S. cerevisiae* adapted to hostile natural conditions form different types of biofilms with a more complex internal organization. These are colony biofilms, which consist of aerial cells and invasive pseudohyphae, and wrinkled biofilms at the solid-liquid interface on plastic. Important questions are how the architecture of these structures is formed and which environmental and regulatory mechanisms determine the formation of differently structured communities.

### Methods

To clarify the internal architecture and identify factors that differentiate biofilm structure from colony structure, we used a spectrum of techniques including proteomics, microscopy and genetically modified yeast strains.

### Results and conclusions

We will present two regulatory pathways that we have recently identified and highlight the specificities of their functions in yeast spatial structures: One involves the regulator *Cyc8p* and is involved in the cellular decision between biofilm and smooth colony lifestyle in response to an environmental signal. The second, involving the membrane-bound *Whi2p-Psr1p/Psr2p* complex, is important for coordinated cellular development in colonies and regulates the expansion of cells with novel properties. Specific links between these regulators and other regulatory and metabolic pathways important for yeast life in differently structured communities will be presented.

This work was supported by GACR23-06368S.

### Keywords:

yeast, differentiated cell subpopulations, regulation

## Competition in biofilms between different *Pseudomonas aeruginosa* clones from cystic fibrosis patients

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### Background

*Pseudomonas aeruginosa* infections are common in cystic fibrosis (CF) patients. Despite continuous exposure to new *P. aeruginosa* clones, most CF patients acquire and retain a single clone in their lungs for decades. This study aims to investigate whether these strains can form mixed biofilms based on their clonal relatedness.

### Methodology

Four *P. aeruginosa* strains isolated from three CF patients were selected for the study. Growth rates, twitching and swarming motility were analysed. Genes encoding cyan (CFP) and yellow (YFP) fluorescent proteins were inserted into the bacterial chromosomes by conjugation. Finally, flowcell systems were inoculated with CFP/YFP tagged strains in a ratio of 1:1 and biofilm formation was investigated. 3D images were acquired at days 3 and 5 by confocal laser scanning microscopy. The image analysis software Imaris was used to process images and do statistics.

### Results

Four biofilms combining two different strains were cultivated. In experiment 1, clonal strains from the sinus and lung of same patient (CF430-142, CF430-11621) were combined. In the rest, non-clonal strains were combined. The strain CF430-11621 was combined with *P. aeruginosa* strains 75887-B (experiment 2) or 8271-B (experiment 3) or with PAO1 strain (experiment 4).

In experiment 1, both strains were able to form biofilms separately. When combined, microcolonies containing a mixture of both clones were observed at day 3. In contrast, no mixture of the clones was observed in the three remaining experiments. In experiment 2, the strain 75887-B had a slow growth and a weak biofilm formation. When looking at the channel inoculated with both strains, only biofilms formed by the strain CF430-11621 were observed. In experiments 3 and 4, all the strains were able to form biofilms separately. When combined, biofilms of both strains were found in the same locations but no mixture of the clones was observed in the microcolonies.

Finally, the strains CF430-142 and 75887-B lacked twitching motility. No correlation between motility and biofilm formation was observed.

### Conclusion

*P. aeruginosa* clonal strains are capable to form mixed biofilms. On the contrary, different clones from *P. aeruginosa* were not able to form mixed biofilms and mono-clonal co-localized biofilms were observed.

### Keywords:

*Pseudomonas aeruginosa*, cystic fibrosis, biofilms

## STRATEGIES FOR FIGHTING BACK BIOFILMS

PG2-29

### Use of a novel peptide strategy to disrupt quorum sensing and biofilms in Gram-negative bacteria

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#### Background and aims

Quorum sensing (QS) is a mechanism by which bacteria can regulate gene expression based on population density. In this respect, bacteria can secrete small signalling molecules (e.g., N-acyl homoserine lactone; AHL) and/or autoinducing peptides (AIPs) that are recognized by intracellular or membrane-bound receptors of neighbouring cells. Upon recognition, AHL and AIPs can up/downregulate various genes in these cells, thus regulating the expression of important phenotypes, such as biofilm formation.

This study aims to develop a novel peptide-based strategy to disrupt QS in bacteria, inhibiting virulence factor biosynthesis and facilitating biofilm dispersion.

#### Methods

Inhibition of the QS systems in *P. aeruginosa* and *E. coli* was investigated using strains containing QS response regulator-reporter gene fusions (for example, *lasR-gfp*) and measuring fluorescence or luminescence. The biosynthesis of *P. aeruginosa* virulence factors pyocyanin and hemolysin, was quantified by Chloroform-HCl and Rabbit blood assays, respectively. Biofilm was quantified by using a crystal violet assay and counting CFU/ml. Furthermore, the direct interaction of peptides with pyocyanin was determined using circular dichroism, and peptide cytotoxicity was examined using a human fibroblast cell line (HFF-1), as per ISO 10993-5:2009.

#### Results

All peptides tested (i.e., buffered and intrinsically acidic analogs), exhibited similar inhibition of the QS response regulators LasR, RhlA, and PqsR (in *P. aeruginosa*), and gene *lasR* (in *E. coli*). Importantly, QS inhibition directly translated into decreased pyocyanin biosynthesis and hemolysin activity in both *P. aeruginosa* reporter strains and clinical isolates. The intrinsically low pH of the peptides tested, also significantly inhibited biofilm formation and triggered the dispersion of pre-established biofilms. Furthermore, all peptide forms (acidic and buffered) directly bound and modulated pyocyanin structure, and inhibited its ability to intercalate with DNA. No cytotoxicity was observed when the peptides were tested using a human fibroblast cell line.

#### Conclusions

To conclude, this study has shown that the peptides tested can disrupt bacterial QS, inhibit virulence factor biosynthesis (and associated biochemical function), and disperse established biofilms. As a consequence, these peptides represent a new treatment strategy for chronic infections, such as those associated with wounds and diabetes-related foot ulcers.

#### Keywords:

Quorum Sensing, Pyocyanin, *Pseudomonas aeruginosa*

## Improving survival in premature infants: development of feeding tubes with a biofilm resistant coating

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### Background

Biofilm formation on the surfaces of medical devices is a leading cause of hospital-acquired infections among premature neonates. Up to 56% of neonatal deaths can be related to these infections and surviving babies are often affected by life-long disabilities. Feeding tubes are among the most widely used medical devices in neonatal intensive care. Up to 89% of feeding tubes removed from infants are colonised by bacteria, in some cases as early as one day after insertion of the device. Feeds provided through contaminated tubes can lead to gastrointestinal or systemic infections, which can ultimately evolve into fatal outcomes. To address this problem preventively, we are developing neonatal feeding tubes with reduced infection risk by employing a biocompatible and non-biocidal anti-biofilm polymer as a coating.

### Methods

Commercially available polyurethane nasogastric paediatric feeding tubes were investigated as coating substrates in this study. Three different co-polymers with optimised mechanical properties were selected among a range of previously identified anti-biofilm materials according to their suitability for coating the substrate, i.e., compatible flexibility and mechanical resistance. The feeding tubes were cut into 1-cm length segments and dip coated into polymer solutions. The coating characteristics were assessed using optical profilometry. Biofilm formation of clinically relevant fluorescent-labelled bacterial species on the samples was investigated with scanning confocal microscopy. The performance of the coated tubes was also assessed under milk conditioning.

### Results

The polymers were successfully demonstrated to form uniform coatings of approximately 20 µm thickness on the substrate, exhibiting good mechanical durability. Tubes coated with poly(isobornyl acrylate-co-diethylene glycol methacrylate) 60:40 demonstrated the best overall bacterial biofilm resistance in vitro, with an average reduction of 89% compared with uncoated tubes for *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa*.

### Conclusion

Our findings indicate potential for the development of a new coated device that prevents biofilm-related infections among high-risk premature infants.

### Keywords:

premature infants, medical devices, biofilms

## Determination of anti-biofilm compound targets across multiple bacterial species

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### Background

Biofilm-mediated bacterial infections are difficult to treat, underscoring the need for a strategy that specifically targets the biofilm state. We previously identified two small molecules, JG-1 and M4, that in vitro, disperse biofilms of *Salmonella Typhimurium* (STm) and *Enterobacter cloacae* (Ec), and that M4 is bactericidal against *Staphylococcus aureus* (Sa). However, the compound targets remain unknown. Our goal was to identify the target with binding assays (bait-prey pulldown [PD] assay, thermal proteome profiling [TPP]), and RNAseq.

### Methods

In the TPP, biofilms (STm and Ec) were incubated with 80  $\mu$ M JG-1, M4, or vehicle (DMSO) and then aliquots of biofilm cells were heated to 25-90°C, expecting that proteins bound to JG-1/M4 are stabilized, thereby increasing their melting temperature. Bacterial proteins were isolated and compared by LC/MS-MS to identify shifts in melting temperatures. For the PD, cell lysates (STm, Sa, and Ec) were incubated with biotinylated JG-1 or M4 bound to a neutravidin column. Bound proteins were visualized via gel electrophoresis and bands unique to the JG-1 or M4 columns were excised and identified by mass spectrometry. RNAseq analysis of biofilms (STm and Ec) and planktonic cells (Sa) treated with JG-1 and/or M4 identified differentially expressed genes by comparison to DMSO. Identified proteins were further examined using single gene deletion mutants (STm and Sa) to test for recalcitrance to JG-1/M4 by comparison to wild-type strain.

### Results

From TPP, multiple proteins in STm and Ec demonstrated increased stability at higher temperatures when incubated with JG-1 or M4. In the PD assays, 30 proteins in STm, 38 proteins in Ec, and 44 proteins in Sa were identified. The RNAseq identified >45 differentially expressed genes and pathway analysis identified multiple enriched pathways, with some overlap between species. Based on the previous results, we tested 84 STm mutants and 23 Sa mutants and identified multiple mutants that confer recalcitrance or increased susceptibility to JG-1 or M4.

### Conclusion

We have identified potential bacterial proteins that when bound to JG-1/M4, result in inhibition of biofilm formation and cell death. Transcriptional pathways were also identified that are important to compound activity, resulting in cell death or biofilm dispersal.

### Keywords:

anti-biofilm agents, *Salmonella*, ESKAPE pathogens

## Carpe pili! Time-lapse monitoring of *Pseudomonas aeruginosa* biofilm eradication by bacteriophage JBD30

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### Background incl. aims

Treatment of infections associated with *Pseudomonas aeruginosa* biofilm is complicated by frequent multi-drug resistance of this bacterium. Bacteriophages are considered as an alternative treatment to antibiotics. Unlike classical therapeutics, phages are complex evolving organisms, and their thorough characterization is required before they can be approved for therapeutic use. Here we employed the combination of cryo-electron microscopy and live cell fluorescence imaging to prepare time-resolved structural model of infection cycle of bacteriophage JBD30 in single cell and in *P. aeruginosa* biofilm.

### Methods

*P. aeruginosa* biofilm was grown in the chamber of light-sheet fluorescent microscope within a unique flow-system enabling continuous imaging and supply of fresh media. After 20 hours of biofilm growth, bacteriophage JBD30 labelled with cyan fluorescent protein fused to major capsid protein C-terminus was introduced into the system. To monitor the dynamics of JBD30 dissemination through the biofilm, z-stacks images were acquired every 15 min.

### Results

The introduction of phage JBD30 to 20 hours old *P. aeruginosa* biofilm led to the complete disruption of the biofilm architecture. Phage tail fibres recognise *P. aeruginosa* pili type IV that grow from the cell poles and are essential for biofilm formation. After pili retraction, tripod of JBD30 receptor binding protein trimers bind to the cell surface lipopolysaccharides. Subsequent conformational changes of JBD30 baseplate lead to the opening of baseplate tip and release of tape measure protein, which forms a channel for DNA ejection through the cell membranes into the host cell. For replication, phage DNA re-distributes from the cell poles through the cytoplasm. Capsids and tails of new JBD30 virions are assembled in separate pathways and then joined together. Replication cycle ends with the lysis of *P. aeruginosa* cell and release of JBD30 progeny 85 min post infection.

### Conclusions

We developed a unique flow-system for cultivation and time-lapse monitoring of *P. aeruginosa* biofilm. Treatment of *P. aeruginosa* biofilm by bacteriophage JBD30 led to complete biofilm eradication. Cryo-EM and light-sheet fluorescence imaging of infected cells and phage particles enabled us to characterize JBD30 replication in *P. aeruginosa* cells and decipher the mechanism of phage JBD30 proliferation through *P. aeruginosa* biofilm.

### Keywords:

*Pseudomonas*, bacteriophage JBD30, fluorescence, cryo-EM

## Nitric oxide donors and ultrasound: a combinational approach to treat chronic wound biofilm infections

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### Background

Bacterial biofilms in chronic wounds are a significant health concern as they disrupt the normal healing process, increasing infection-associated morbidity and mortality. Previous research has explored nitric oxide (NO) gas as an antibiofilm agent, but its clinical applicability is limited due to its short half-life and fast diffusion in vivo. Ultrasound-responsive NO-loaded microbubbles (MBs) have also shown promising antibiofilm effects but also present challenges including burst release. This study evaluated pH-dependent NO donors, PAPA NONOate (PA-NO) and spermine NONOate (SP-NO) for integration into an ultrasound-responsive system. The aims were to investigate the pH-dependence of NONOate decomposition, assess the antibiofilm effects of NONOate-only treatment in wound-relevant conditions, and evaluate how perfluorobutane-core MBs (PFBMBs) affect biofilms at varying ultrasound pressures.

### Methods

For crystal violet (CV) studies, *Pseudomonas aeruginosa* (PAO1) biofilms were grown for 24 hours at 37 °C in 96-well plates. Treatment was applied for 60 or 120 min at 32 °C and then stained with CV. The absorbance of CV-stained biofilm was measured in a plate reader. NONOate degradation studies measured changes in absorbance at 250 and 252 nm. For ultrasound studies, ultrasound was applied at 1.1 MHz driving frequency, 20% duty cycle and 20 Hz pulse-repetition-frequency, for 40 seconds. The biofilm was stained using SYTO9.

### Results

For NONOate-only treatment, significant biofilm dispersal was observed after 120 minutes of treatment with PA-NO at pH 5.5, 7.5, and 8.5, and pH 7.5 for SP-NO at 32 °C. The most significant dispersal was observed at pH 7.5, with biomass reductions of ~55% and ~45% for PA-NO and SP-NO, respectively. The degradation profile of NONOates was optimal at pH 7.5, showing sustained NO release throughout the treatment period. Biofilms treated with PFBMBs-only showed distinct dispersal profiles at varying acoustic pressures (0.5-2.0 MPa).

### Conclusions

The results suggest that NONOates could serve as an antibiofilm therapy when integrated into an ultrasound-responsive system. The study assessed antibiofilm effects in wound-relevant conditions, considering pH and temperature for the first time. Ongoing research aims to explore the combined effects of NONOates, antibiotics, and PFBMBs for treating chronic wound biofilms, providing a potential avenue for improved therapeutic strategies.

### Keywords:

Nitric oxide ultrasound biofilm wound

## Plasma activated water as a pre-treatment strategy in the context of biofilm-infected chronic wounds

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Healing and treatment of chronic wounds are often complicated due to biofilm formation by pathogens. Here, the efficacy of plasma activated water (PAW) as a pre-treatment strategy has been investigated prior to the application of topical antiseptics polyhexamethylene biguanide, povidone iodine, and MediHoney, which are routinely used to treat chronic wounds. The efficacy of this treatment strategy was determined against biofilms of *Escherichia coli* formed on a plastic substratum and on a human keratinocyte monolayer substratum used as an in vitro biofilm-skin epithelial cell model. PAW pre-treatment greatly increased the killing efficacy of all the three antiseptics to eradicate the *E. coli* biofilms formed on the plastic and keratinocyte substrates. However, the efficacy of the combined PAW-antiseptic treatment and single treatments using PAW or antiseptic alone was lower for biofilms formed in the in vitro biofilm-skin epithelial cell model compared to the plastic substratum. Scavenging assays demonstrated that reactive species present within the PAW were largely responsible for its anti-biofilm activity. PAW treatment resulted in significant intracellular reactive oxygen and nitrogen species accumulation within the *E. coli* biofilms, while also rapidly acting on the microbial membrane leading to outer membrane permeabilisation and depolarisation. Together, these factors contribute to significant cell death, potentiating the antibacterial effect of the assessed antiseptics.

### **Keywords:**

Plasma; Wounds; Antiseptics; *Escherichia coli*

## Innovative decontamination of Salmonella Typhimurium biofilms by plasma-activated water

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### Background

In the EU, over 91,000 cases of Salmonella outbreaks are reported each year, costing the economy and public health system €3 billion annually. There is an imminent need to improve food sanitisation. The application of plasma-activated water (PAW) in the food industry has emerged as a promising approach to enhance food safety. PAW is water that has been treated with cold plasma, which enriches the water with reactive oxygen and nitrogen species, enhancing its antimicrobial properties. This study aimed to evaluate the efficacy of different types of PAW against Salmonella biofilms.

### Methods

We investigated the impact of PAW on biofilm formation, cell viability, and biofilm disruption using Salmonella Typhimurium isolates. Time-kill assays were employed to determine bactericidal effects. The ability of PAW to inhibit bacterial invasion was assessed using polarised Caco-2 cell models. The Bioflux system was utilised to examine biofilm eradication and prevention of regrowth post-treatment. The ability of PAW to remove established Salmonella biofilms from contaminated eggshells was tested. Ultrastructural changes induced by PAW on Salmonella and eggshells were visualised using scanning and transmission electron microscopy (SEM/TEM).

### Results

PAW demonstrated potent antibacterial activity against all tested Salmonella Typhimurium isolates. It showed superior bactericidal capacity, significantly reducing Salmonella viability and completely inhibiting bacterial invasion into host cells. Bioflux assays confirmed the complete eradication of Salmonella biofilms by PAW, with no regrowth observed up to 72 hours post-removal. A 2 log<sub>10</sub> CFU reduction in biofilm biomass on eggshells was achieved with PAW. SEM analysis indicated that PAW treatment preserved eggshell integrity, while TEM analysis revealed PAW-induced membrane damage in Salmonella, characterised by shrinkage and leakage, leading to bacterial cell death.

### Conclusions

PAW has demonstrated significant antibacterial efficacy against Salmonella Typhimurium, including the ability to inhibit bacterial invasion, disrupt biofilms, and reduce bacterial viability. These findings underscore the potential of PAW as an effective sanitising agent in the food industry, offering a promising solution for reducing Salmonella contamination to enhance food safety and public health.

### Keywords:

Cold-Plasma, Sanitisation, Salmonella, Innovative Therapeutics

## Efficacy and host response of different wound debridement strategies

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### Background

Biofilm infections have challenged the biomedical community to develop novel treatment strategies. To minimize patient discomfort and increase accessibility, wound debridement has expanded beyond surgical removal of infected tissue to include numerous enzymatic therapeutics for degrading necrotic tissue. However, these emerging treatments have also presented with new risks, particularly increased concerns of bacteremia and sepsis. We investigated various debridement strategies in our pre-clinical murine surgical wound model. Our aims were to elucidate biofilm dispersal efficacy and host response to each debridement strategy. We hypothesized that enzymatic debridement strategies with high levels of biofilm dispersal and tissue degradation would leave the host vulnerable to systemic infection spread with elevated risk of bacteremia and sepsis.

### Methods

We evaluated the efficacy of debridement strategies on *Pseudomonas aeruginosa* (PA) infections in our murine surgical wound model. Dorsal 1.5cm<sup>2</sup> full-thickness surgical incisions were inoculated with PAO1. Infected wounds were treated with various debridement strategies, including sharp debridement, glycoside hydrolases (GH), and other clinically relevant enzymes on days 2 and 5 post-wound and infection. Dispersal efficacy was assessed ex vivo and host response, including bacteremia and sepsis, was assessed in vivo.

### Results

Our results suggest that GH enzymatic debridement has high biofilm dispersal efficacy and is associated with a marked increase in incidence of bacteremia and sepsis compared to other clinically utilized debridement strategies, such as sharp debridement. Furthermore, we determined that while biofilm dispersal efficacy remains high, risk of systemic infection was dramatically reduced for older wound infections.

### Conclusions

This study provides valuable insight into the efficacy and potential risks associated with several clinically relevant debridement strategies. Our findings indicate the importance of tailoring treatment based on wound and infection characteristics to maximize biofilm disruption while minimizing risks to patient health. Future studies will focus the bacterial response to biofilm dispersal in wound infections.

### Keywords:

Dispersal, debridement, therapeutics, wounds

## Measuring the Nitric Oxide Capacity of Perfluoropentane Droplets for the Treatment of Chronic Wound Biofilms

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### Background

Perfluorocarbon droplets (PDs) are promising particles for antimicrobial interventions, particularly in the treatment of biofilm-associated infections. Nitric oxide (NO) is known to induce biofilm dispersal, although an extremely short half-life makes delivery difficult. The liquid perfluorocarbon core of PDs can be stabilised by a lipid shell and can dissolve bioactive gases such as NO. PDs are responsive to ultrasound waves and can be converted into gas-filled microbubbles through acoustic droplet vaporization (ADV). Subsequent microbubble cavitation can deliver mechanical disruption or allow for temporal control in the release of loaded therapeutic molecules. The aim of this study is to quantify the NO-capacity of PD emulsions. Subsequently ADV thresholds will be determined, and PDs will be tested on biofilm models.

### Methods

Droplets were manufactured by a 2-step sonication process, emulsifying a lipid solution with perfluoro-n-pentane (PFP). Initially, 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC) and polyoxyethylene (40) stearate (PEG40S) were dispersed in DPBS in a 9:1 molar ratio. PFP was added in a 9:1 or 4:1 Lipid:PFP ratio (10% and 20% v/v PFP, respectively) prior to the second sonication step. Pulsed sonication (2 s on-15 s off, 60 s total sonication time) was used to make PDs within a water-ice bath to maintain sample temperature <15 °C, necessary due to the low boiling point of bulk PFP (28 °C).

An oxygen-free NO-source (400-ppm NO in nitrogen) was flowed over samples for 2 min before sealing for 2h on ice. MilliQ water, Lipids-only, 10% and 20% v/v PFP droplet samples were NO-loaded. NO-oxide (NOx) concentrations were detected using a chemiluminescence NO analyzer (CLD 88, ECO MEDICS AG).

### Results

NOx concentrations were measured in water, lipids and both 10% and 20% v/v PFP PD samples. The mean NOx concentrations ( $\pm 1$  S.D.) for NO-water, NO-lipids, NO-PDs (10% v/v) and NO-PDs (20% v/v) were  $1.89 \pm 0.66 \mu\text{M}$ ,  $2.76 \pm 0.88 \mu\text{M}$ ,  $4.34 \pm 0.57 \mu\text{M}$  and  $4.61 \pm 0.32 \mu\text{M}$ , respectively.

### Conclusions

PFP-core droplets were shown to carry significantly more NOx than lipids or water alone. PDs show potential as bioactive gas carriers for delivery to biofilms. However, increasing the volumetric concentration of PFP did not significantly increase NOx concentration.

### Keywords:

Nitric Oxide, Perfluorocarbon droplets, Ultrasound

## Antibacterial and anti-biofilm activities of antibiotic-free phosphatidylglycerol/docosahexaenoic acid lamellar and non-lamellar liquid crystalline nanoparticles

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### Background

Docosahexaenoic acid (DHA) has the potential to combat microbial infections and phosphatidylglycerol (DOPG) is attractive for use as a biocompatible prominent amphiphilic component of the cell membrane of Gram-positive bacteria. In the present study, we aim to assess the anti-bacterial and anti-biofilm activities of antibiotic-free nanoformulations (hexosomes and liposomes) produced from binary mixtures of DHA and DOPG against *S. aureus* ATCC 29213 and *S. epidermidis* 5D, which are frequent causes of infections on catheters and medical devices.

### Methods

In addition to biophysical characterization investigations, the anti-bacterial and anti-biofilm activities were evaluated through the use of different techniques, including resazurin assay, time-kill assay, and confocal laser scanning microscopy (CLSM) on an in vitro biofilm model.

### Results & conclusions

In addition to biophysical characterization studies, the antibacterial activity of two different DOPG/DHA nanoformulations (hexosomes DOPG:DHA 1:1 and liposomes DOPG:DHA 2:3) has been evaluated through the use of resazurin assay. The MICs of the hexosomes and liposomes were 0.62 mg/ml against *S. aureus* and 12.5 mg/ml against *S. epidermidis*. Further, the time-kill curves show the killing of the *S. aureus* population (from 10<sup>6</sup> CFU/ml to 0) after the first 2-4 hours of incubation with liposomes and hexosomes, probably attributed to the interaction of the nanoparticles with the bacterial cell membrane. Both nanoformulations had a bacteriostatic effect on *S. epidermidis* 5D in the first 8 hours. Using CLSM, the viability of *S. aureus* gfp AH 2547 biofilms on catheters, treated with liposomes, was evaluated. Liposomes completely killed the 24-h biofilm formed by *S. aureus* ATCC 29213; whereas hexosomes significantly reduced the biofilms of *S. aureus* ATCC 29213 and *S. aureus* gfp AH 2547. There was no anti-biofilm activity against *S. epidermidis*. This may be attributed to the difference in the biofilm composition formed by the two species.

### Keywords:

Docosahexaenoic acid, hexosomes, biofilm, liposomes

## Ultrasound-responsive nanoscale delivery platform for the treatment of biofilm-associated diseases

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### Background

Many antibiofilm agents are unable to efficiently accumulate beyond the biofilm matrix or are retained poorly within it, leading to sub-therapeutic concentrations within the biofilm. To address this fundamental delivery constraint, we have established an ultrasound-responsive nanoscale drug delivery platform capable of spatiotemporally controlled drug release and simultaneously promoting biofilm disruption.

### Methods

We explore the stable inclusion of four antimicrobials, each with a different mechanism of action, within an adapted condensed clinical ultrasound contrast agent – azithromycin, besifloxacin, polymyxin B, and ruthenium. Minimum planktonic, biofilm, and persister cell bactericidal concentrations were assessed using fluorometric and microdilution methods across 10 clinical isolates of Gram+ and Gram- species. The translatability of the platform was explored under flow using artificial clinically-relevant medias. To validate the mechanism of action, picomolar-level cellular and subcellular uptake was assayed using europium-chelates of antimicrobials alongside simultaneous ultra-high-speed imaging and fluorescence microscopy to visualize real-time biofilm disruption.

### Results

High-yield serum-stable 150-250 nm nanodroplets were formed and successfully loaded with all four tested antimicrobial agents. Nanodroplets showed a strong protective effect against planktonic bacteria across all measures, preventing premature release of drug and unnecessary destruction of the microbiome. When stimulated with therapeutic ultrasound however, an average of 22.8x lower antimicrobial concentrations were needed to achieve 99.9% biofilm eradication by viability and 167.8x lower by biomass. To eradicate all cells, including persisters and those dormant, an average of 13.8x lower concentration of the different antimicrobials was needed. Evaluating cellular and subcellular uptake of drug within both planktonic bacteria and biofilms suggests that much of the concentration-dependent effects observed may be attributable to the preferential uptake beyond the biofilm matrix into the bacterial cell, and specifically within membrane-bound organelles. Combined with visualization of biofilm disruption following ultrasound exposure, these studies present a promising opportunity to efficaciously deliver antimicrobial agents to clinical biofilm infections.

### Conclusions

Here, we introduce an ultrasound-responsive delivery strategy to deliver antimicrobials beyond the biofilm matrix for spatiotemporally controlled antibiofilm therapy. We hope the findings and framework of this study will serve to guide the design of novel mechanosensitive drug delivery platforms to further their potential for clinical implementation.

### Keywords:

Delivery, Nanomedicine, Ultrasound, Disruption, Persister

PG2-41

## What's the fate of prosthetic joint infection-associated cutibacterium acnes in the battle with neutrophils?

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Cutibacterium acnes causes prosthetic joint infections (PJIs) by forming resilient biofilms on prosthesis, potentially evading host defense mechanisms. While our previous findings indicate that polymorphonuclear neutrophils (PMNs), the "first line of defense" against bacterial infection, can recognize biofilms-associated and planktonic *C. acnes*, this study aims to determine PMNs' efficacy in eliminating biofilm-embedded and planktonic *C. acnes*, shedding light on the persistence of *C. acnes*-associated PJI.

### **Keywords:**

Cutibacterium, prosthetic joint infection, biofilm

## Effect of *Alkanna tinctoria* against MRSA and *Pseudomonas aeruginosa* biofilms on excision wound in diabetic mice

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*Alkanna tinctoria* commonly called as dyer's alkanet (family- Boraginaceae) is used in Saudi folk medicine to treat skin infections. A methanolic extract and a traditional formulation of the plant used in folklore were prepared. The phytoconstituents present in both the extract and the traditional formulation were determined using LC-MS. In-vitro and in-vivo antibacterial and antibiofilm activities of were evaluated against multidrug-resistant bacteria. Cytotoxic effect was evaluated using HaCaT cell lines in-vitro. The in-vivo antibiofilm and wound healing activity was evaluated in diabetic mice using the excision wound method. More than 50 different phytoconstituents were found in the methanolic extract and more than 40 chemical constituents were present in the traditional formulation. The extract ointment exhibited antibacterial activity against both the tested pathogens, but it had skin irritation effect on the mouse skin. However, the traditional formulation was an effective antibacterial agent and was free of skin irritant effect. The methanolic extract was cytotoxic on HaCaT cells while the traditional formulation did not show any cytotoxic effect. The methanolic extract ointment decreased the healing of wounds whereas the traditional formulation accelerated the wound healing in diabetic mice. The results revealed that *Alkanna tinctoria* in its traditional formulation is an effective wound healing agent but the methanolic extract of the plant does not increase healing of wounds due to its skin irritation effect.

### **Keywords:**

Biofilm; LCMS analysis; cytotoxicity; epithelization

## Inhaled aztreonam-tobramycin combination provides synergistic effects against planktonic and biofilm states of hypermutable *Pseudomonas aeruginosa* isolates from patients with cystic fibrosis

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### Background

Hypermutable *Pseudomonas aeruginosa* strains are highly prevalent in chronic lung infections of patients with cystic fibrosis. Acute exacerbations of these infections have limited treatment options. This study aimed to investigate inhaled aztreonam and tobramycin against clinical hypermutable *P. aeruginosa* strains using the CDC (Centers for Disease Control and Prevention) dynamic in vitro biofilm reactor, mechanism-based mathematical modelling, and genomic studies.

### Methods

Two cystic fibrosis multidrug-resistant strains were investigated in 168-h dynamic in vitro biofilm studies (n=2 biological replicates). Regimens were inhaled aztreonam (75 mg 8-hourly) and tobramycin (300 mg 12-hourly) in monotherapies and combination. The simulated pharmacokinetic profiles of aztreonam and tobramycin (elimination half-life 3h) were based on published lung fluid concentrations in patients with cystic fibrosis. Total viable and resistant counts were determined for planktonic and biofilm bacteria. Mechanism-based modelling of total and resistant bacterial counts, and whole genome sequencing were completed.

### Results

Both isolates showed reproducible bacterial regrowth and resistance amplification for the monotherapies by 168h. The combination performed synergistically, with minimal resistant subpopulations compared to the respective monotherapies at 168h. Mechanistic synergy appropriately described the antibacterial effects of the combination regimen in the mechanism-based model. Genomic analysis of colonies recovered from monotherapy regimens indicated noncanonical resistance mechanisms were likely responsible for treatment failure.

### Conclusions

The combination of aztreonam and tobramycin was required to suppress regrowth and resistance of planktonic and biofilm bacteria in all biological replicates of both hypermutable multidrug-resistant *P. aeruginosa* cystic fibrosis isolates. The developed mechanism-based model could be utilised for future investigations of this promising inhaled combination.

### Keywords:

hypermutator, *Pseudomonas*, mathematical modelling, sequencing

## Identification of Genes Essential for Colonisation of Uropathogenic *Escherichia coli* (UPEC) on a Probiotic Biofilm Pre-Established on Urinary Catheters

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Catheter associated urinary tract infections (CAUTIs) are frequently caused by pathogens such as uropathogenic *Escherichia coli* (UPEC) which adhere to the catheter's lumen and form biofilms before ascending into the bladder and kidneys. Catheters with a preformed probiotic biofilm have previously been shown to prevent the colonisation of urinary pathogens in a static incubation setting. A clinical trial showed probiotic strains, *Limosilactobacillus reuteri* RC14 and *Lactisacibacillus rhamnosus* GR1, have benefits in protecting against colonisation leading to CAUTIs in spinal cord injury patients when taken orally.

Here a dual species biofilm of *L. reuteri* RC14 and *L. rhamnosus* GR1 was established on catheters and challenged by introduction of UPEC strains in both static and flow conditions. The colonisation by UPEC strains were quantified by plate counts after 3 and 48 hours of infection to measure the initial adherence and long-term survival of the UPEC strains in presence of the pre-established biofilm. Transposon directed insertion sequencing (TraDIS) was used to determine essential genes required for adherence and survival of the UPEC in these biofilms. UPEC transposon libraries were introduced to the already established dual probiotic biofilm and attached cells were sequenced after 3 and 48 hours.

The in-vitro incubation conditions of the catheter significantly impacted the probiotics' ability to inhibit UPEC colonisation. In static 24-well plates, UPEC were completely inhibited from colonising catheters with a preformed probiotic biofilm whilst under flow conditions no inhibition was observed. Furthermore, we showed complete inhibition of UPEC strains by using cell free supernatant of the probiotics strains due to inhibitory effect of well-known probiotic secreted secondary metabolites. TraDIS revealed genes associated with amino acid synthesis, metabolism and motility were essential for adherence and survival of UPEC strains in the probiotic biofilm. This work demonstrated the importance of flow in-vitro models when screening for potential probiotics for preventing pathogen colonisation in catheters to better represent the catheterised urinary environment. In addition, identification of gene associated with the adherence and survival of UPEC in probiotic biofilms can be used as target for preventative treatments.

### **Keywords:**

Probiotics, UPEC, CAUTIs, transposon sequencing

## Cell-free supernatants from *Lactocaseibacillus rhamnosus* exert strong antibacterial activity against *Pseudomonas aeruginosa* and *Staphylococcus aureus* mono- and dual-species biofilms in an in vivo-like wound infection model

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### Background

Chronic non-healing wounds are a prevalent medical problem affecting millions of patients across the world. Microbial biofilms are known to contribute significantly to the persistence of infections and the impaired healing of chronic wounds. In the era of microbial resistance, there is growing interest in exploring the potential of probiotic strains and their metabolic products, also referred to as postbiotics, as novel biotherapeutic approaches for the management of chronic, hard-to-heal, wounds. This study aimed to investigate the efficacy of cell-free supernatants (CFS) derived from *Lactocaseibacillus rhamnosus* against mono and dual-species biofilms of wound pathogens such as *Staphylococcus aureus* and *Pseudomonas aeruginosa* using a 3D in vitro model resembling the conditions found in an infected wound.

### Methods

Mono- and dual-species mature biofilms of wound isolates of *P. aeruginosa* and *S. aureus* were established on collagen scaffolds in the presence of a simulant wound fluid (SWF), and treated with CFS at varying doses (1:4 to 1:8 dilution) and time intervals (8, 16, and 24 h). Anti-biofilm activity was assessed by colony-forming unit (CFU) count and/or scanning electron microscopy imaging.

### Results

The results revealed that at a 1:4 dilution in SWF, CFS exhibited a strong ability to reduce the CFU numbers of bacteria within mono-species biofilms, as well as of bacteria released by the biofilms into the supernatant. Additionally, both CFU count and electron microscopy imaging demonstrated a marked antibiofilm effect against dual-species biofilms, which was evident already after 8 h of incubation. The cytotoxic effect of CFS at 24 h against HaCaT cells was acceptable, and notably, CFS did not induce resistance, evaluated as increase in minimum inhibitory concentration (MIC) after 15 passages at sub-inhibitory concentrations. In contrast, the MIC of ciprofloxacin increased 64 and 512 fold against *P. aeruginosa* and *S. aureus*, respectively.

### Conclusions

Overall, these results strengthen the current view that probiotic-derived substances (i.e., postbiotics), may represent promising alternatives to antibiotics, and underscore the potential of *L. rhamnosus* postbiotics as a promising strategy for the treatment of wound biofilms.

### Keywords:

*lactobacilli*, resistance, wound-infection-model, postbiotics, cell-free-supernatants

## Study of AMR-profile, biofilm formation, and phage sensitivity and biofilm-eradication ability in a collection of *K. pneumoniae* carbapenemase-positive UTI strains

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### Background

*Klebsiella pneumoniae* is the second most common cause of urinary tract infections (UTIs) worldwide. Biofilm formation and hypermucoviscosity are associated with increased persistence on urinary epithelial surfaces. Phage therapy has gained interest as a possible alternative against emerging clinical multidrug-resistant (MDR) strains. Therefore, understanding the persistence factors of UTI strains and their relationship with phage susceptibility is of great interest for the development of new treatments.

### Methods

A collection of 24 community/nosocomially acquired *K. pneumoniae* carbapenemase-positive UTI strains were selected to test the lytic capacity of 6 wastewater-derived preselected wide-spectrum lytic phages. Bacteria and phages were sequenced using Illumina. Bacteria were subjected to multi-locus sequence typing (MLST), drug resistance gene identification and phenotypic antimicrobial resistance (AMR) using the NEG-MIC-57 Microscan-panel (c58012). Principal Component Analysis (PCA) was performed for MDR-phenotypic/genotypic data. Hipermucoviscous (HMV) phenotype and biofilm formation (Active Attachment cultivation model) of all strains were assessed in LB supplemented with glucose (0.4%) in microaerobiosis. Additionally, the biofilm eradication ability of one phage in a 24h pre-formed biofilm was studied.

### Results

The 24 UTI strains were identified in ST-15 (n=11), ST-147 (n=8), ST-17, ST-307, ST-392, ST-449 and ST-1961, and 75% of strains showed MDR. PCA analysis of AMR-data showed ST-147 as a separated group. Additionally, 15 strains showed HMV phenotype, 7 out of 8 from ST-147, and 13 were nosocomially-acquired. Regarding biofilm-formation, a high variability was observed between nosocomially/community-acquired strains independently of the ST-groups and HMV-phenotype. The 6 phages belonging to genus Webervirus (n=2), Jiadovirus (n=3), and Drulisvirus (n=1), showed a broad lytic-spectrum activity against UTI-MDR strains. Phage 33D1 (Webervirus) showed the highest lytic spectrum, being active against 15 different strains of 7 different STs, including hyper- and low-biofilm formers. Interestingly, HMV-negative strains showed higher levels of susceptibility to phages. Moreover, phage 33D1 showed biofilm eradication ability against 24h preformed biofilms, reducing 2-log<sub>10</sub> biofilm cells.

### Conclusions

Considering the high variability observed in biofilm formation, and the high-extended lytic-spectrum activity against the UTI-MDR strains of the tested phages, plus the confirmed eradication ability of 33D1, our results suggest that phage-therapy may be suitable for development of new UTI treatments.

### Keywords:

Biofilm, phage, AMR, UTI, HMV

## Targeting mucosal gastrointestinal biofilms with antimicrobial peptides

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Gastrointestinal (GI) disorders, such as inflammatory bowel diseases (IBD) and irritable bowel syndrome (IBS) affect ~6.8 million and ~780 million individuals globally, respectively. These diseases pose significant global challenges and substantially burden public health and the economy worldwide. Mucosal-associated biofilms are highly prevalent in the GI tracts of IBS and IBD patients. Preliminary clinical work in removing these biofilms through endoscopic jet washes appears to reduce the functional GI symptoms experienced by the patients. Currently, no pharmaceutical interventions exist targeting such GI biofilms. Hence, there is an unmet need to develop oral therapeutics to target biofilms associated with gut disorders.

Antimicrobial peptides (AMPs), identified in various animals and humans, effectively defend against bacteria. We aim to explore other AMPs to develop oral therapeutics for biofilm-associated GI diseases. We selected AMP candidates by reviewing the literature and specific AMP databases. We synthesised the peptides by solid-phase peptide synthesis and screened them to assess their antimicrobial and antibiofilm properties. We have successfully synthesised, purified, and screened 30 AMPs from various organisms (amphibians, marsupials, and insects) for antimicrobial and antibiofilm activity against 2 biofilm-positive patient isolates of *Streptococcus parasanguinis* 102-K3/3 and *Escherichia coli* 104-K1 and 2 literature-control strains of *Staphylococcus aureus* ATCC 29213 and *Pseudomonas aeruginosa* ATCC 27853. Ongoing efforts involve investigating the other AMPs, expanding to additional clinically isolated GI biofilm-producing strains, conducting structure-activity relationship studies of identified leads, and improving their gut stability for oral administration. These advancements are crucial for their potential application in treating biofilm-positive patients with GI diseases.

### **Keywords:**

antibiofilms, antimicrobials, peptides, gastrointestinal biofilms

## Exploiting bacteriophage endoglycosidase against *K. pneumoniae* biofilms

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### Background information

*Klebsiella pneumoniae* (Kp) is a Gram-negative opportunistic pathogen considered as one of the most critical bacterial species by several health organizations. Infections by multi drug resistant (MDR) Kp represent a major clinical challenge, given their massive spread in hospital settings and the complexity of their treatment due to the lack of effective drugs. The capsular polysaccharide (CPS) is an important virulence factor and it is also a major component of bacterial biofilms, thus representing an interesting target for the development of alternative antimicrobial strategies. The CPS differs among *K. pneumoniae* strains in terms of type of sugars and glycosidic linkages and about 80 different structures are known, therefore it can be considered a very specific target. This study aims to characterize the activity of the endoglycosidase belonging to the bacteriophage phiBO1E, specific for the CPS of *K. pneumoniae* KpB-1. This strain is of particular interest, since it belongs to the Clonal Group 258, one of the main responsible for the worldwide pandemic.

### Methods

To test the phage endoglycosidase activity on the CPS, phage particles were incubated with pure CPS, the products were separated by size exclusion chromatography and analysed by NMR spectroscopy and ESI-mass spectrometry. To test the potential anti-biofilm activity of phiBO1E, the preformed biofilm was treated with different phage concentrations. The phage endoglycosidase gene was cloned and expressed in *E. coli*, then the protein was purified by immobilized metal affinity chromatography and characterized by Circular Dichroism spectroscopy.

### Results

Analysis of pure CPS after incubation with phage-associated endoglycosidase determined the endoglycosidase cleavage site.

Treatment of preformed KpB-1 biofilm with phage particles ( $1 \times 10^9$  PFU/mL) resulted in significant biomass disruption.

The pure endoglycosidase was efficiently obtained and preliminary characterization indicated abundant  $\beta$ -sheet structure.

### Conclusions

The reported preliminary studies confirmed that phiBO1E possesses an endoglycosidase which hydrolyses KpB-1 CPS and biofilm. These data suggest that the purified enzyme, loaded on a carrier together with antibacterial agents, could be used to target selectively *K. pneumoniae* KpB-1, without affecting the healthy microbiota.

### Keywords:

Kp, capsular polysaccharide, biofilm, bacteriophage

## Self-regulating antimicrobial coatings for the treatment of nosocomial infections on catheters

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Bacterial resistance to conventional antibiotics combined with the essential role of biofilms in nosocomial infections caused by medical devices has led to a growing interest in new antimicrobial strategies. Since the formation of bacterial resistances represents a permanent risk during biofilm treatment, especially the prophylactic release of antibiotics has to be avoided. Therefore, it is desirable to prevent the adhesion of microorganisms before the formation of a mature biofilm takes place. Aim of the study was to develop a flexible and self-regulated drug release coating triggered by the presence of low concentrations of microorganisms. For this purpose, different hydrogel matrices based on different ratios of gelatin and poly(lactic-co-glycolic acid) (PLGA) were modified for a stable application in the body. Clinically relevant microorganisms like *Staphylococcus* and *Pseudomonas* species were then tested for their ability to degrade these biopolymers enzymatically. By adapting the modified hydrogels to the enzymatic degradation capacity of the pathogenic microorganisms a smart coating was build where the integration of an antimicrobial therapy option was the next step. For this purpose, model substances were incorporated into the hydrogels. For the investigation of the drug release profile neutral red was incorporated and for investigating the antimicrobial effect incorporated gentamycine was used. The gel solutions were then dip-coated onto conventional catheter materials to form effective layer thicknesses. They were cured and tested in a catheter-associated urinary tract infection model. *Staphylococcus aureus* in particular showed sensitive behavior to the developed drug release mechanism by autonomous hydrolysis of the modified hydrogels. Biofilm formation was thus avoided over a period  $t$  of  $t = 10$  days. Using this approach, an on-demand antimicrobial treatment for emerging nosocomial urinary tract infections was achieved. The results offer great potential for reducing biofilm formation on medical devices, such as catheters or antimicrobial wound dressings. However, the commercial implementation of this technology on a large scale requires further investigation.

### **Keywords:**

antimicrobial coating, drug release, on-demand

## Investigation of the effect of AgNPs-based nanocomposites on *Pseudomonas aeruginosa* biofilm formation under dynamic conditions – Revealing Challenges in Antimicrobial Research

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### Background

Although medical devices, whether implanted or not, are intended to ensure well-being, they may turn into a reservoir for the colonization and growth of microorganisms, thus supporting the onset of a resilient biofilm and ultimately contributing to serious infections. To address this challenge, advancing the development of medical devices with anti-biofilm properties is considered a promising approach. Here, we explore nanocomposite materials based on silver nanoparticles (AgNPs) deposited on the surface of a thin silica (SiO<sub>2</sub>) layers for their potential effect on the adhesion, detachment, viability and biofilm formation of *Pseudomonas aeruginosa*.

### Methods

AgNPs-based nanocomposite thin films were prepared after Ag sputtering on thin SiO<sub>2</sub> surfaces, thermally grown on Si-substrates. The average size of deposited AgNPs was 20 nm, with interparticle distance of 5 nm. The effect of these exposed AgNPs on the adhesion, viability and biofilm formation of *P. aeruginosa* PAO1-Tn7-gfp was evaluated under dynamic conditions, using a shear-stress flow chamber under conditions similar to those encountered around certain medical devices. In the 3-day biofilm experiments, a low-nutritive medium was used to create stressful conditions and promote biofilm formation and growth of adherent cells rather than planktonic growth. The established biofilms were characterized through epifluorescence and atomic force microscopy analyses.

### Results

Findings revealed a rapid bactericidal effect of AgNPs, noticeable within 30 minutes of exposure. Moreover, the presence of some residual viable cells leads to delayed surface colonization for this nanocomposite with no structured biofilm, even after 3 days of dynamic culture, in comparison to SiO<sub>2</sub>. Interestingly, the reinoculation of the potentially persistent *P. aeruginosa* population exhibited flexibility, allowing it to proliferate more rapidly on fresh AgNPs-based nanocomposites than in the first round, although the biofilm structure was different compared to SiO<sub>2</sub> biofilm. The significant reduction in biomass and biofilm alteration upon AgNPs contact needs more exploration to determine whether these effects are correlated with an increase in bacterial sensitivity.

### Conclusion

This study underlines the promising potential of AgNPs coating in the conception of novel antimicrobial/antibiofilm surfaces. Simultaneously, it spotlights the possible adaptation of *P. aeruginosa* PAO1-Tn7-gfp, emphasizing the necessity for future research to address this phenomenon.

### Keywords:

AgNPs, flow chamber, antimicrobial/antibiofilm surfaces.

## Culture filtrate supernatants of *Lacticaseibacillus rhamnosus* exert marked activity against persister cells of *Pseudomonas aeruginosa* under conditions relevant to cystic fibrosis lung

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### Introduction

Persister cells, a subpopulation of bacteria highly tolerant to antibiotics, are recognized for their crucial role in infection relapses and biofilm resilience to antibiotic treatment. Their role is particularly relevant in patients with cystic fibrosis (CF) for which *Pseudomonas aeruginosa* is a major lung pathogen. In the era of antimicrobial resistance, probiotics or their metabolic products are gaining a keen interest as novel antimicrobial strategies. This study explores the anti-persister activity of *Lacticaseibacillus rhamnosus* culture filtrate supernatants (LR-CFS) against *P. aeruginosa* in an artificial sputum medium (ASM) that mimics the CF lung environment.

### Materials and methods

Two lung isolates of *P. aeruginosa* from CF patients exhibiting non-mucoid and mucoid phenotypes respectively were used in the study. Persisters of *P. aeruginosa* in planktonic form were obtained through exposure of stationary phase cultures to cyanide m-chlorophenylhydrazine (CCCP) in LB, or to high doses of tobramycin in ASM. Biofilm-associated persisters were obtained by treating 48h-old biofilms of *P. aeruginosa* with high doses of ciprofloxacin in ASM. The killing effect of LR-CFS against both planktonic and biofilm-associated *P. aeruginosa* persisters was evaluated in ASM by CFU count.

### Results

When pretreated with CCCP, *P. aeruginosa* displayed very low susceptibility to high doses of ciprofloxacin in ASM, while it remained highly susceptible to LR-CFS. These caused a decrease in the CFU number of persister cells of 1 to 3 logs when used at the dilutions of 1:8, 1:6 and 1:4. Exposure of *P. aeruginosa* cultures to high doses of tobramycin for 24h yielded a population of bacteria highly tolerant to a subsequent 24h exposure to the same antibiotic, while 24h exposure to LR-CFS at 1:4 and 1:6 dilution showed a significant bactericidal efficacy. Lastly, biofilm-associated persister cells were successfully obtained, as demonstrated by their recalcitrance to a 24h exposure to ciprofloxacin. A 1-2 log reduction was observed when the same biofilm-associated persister cells were exposed to 1:4 LR-CFS for 24 h.

### Conclusion

The results obtained suggest that metabolites released by *L. rhamnosus* into culture supernatants during growth have the potential to target persister cells of *P. aeruginosa* in conditions mimicking the CF environment.

### Keywords:

Persisters; CysticFibrosis; Biofilm; Probiotics, *P.aeruginosa*.

## Modulation of Streptococcus mutans biofilms by small effector molecules produced by Lactobacillus species

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### Background

Oral microbiome is a highly complex microbial community residing in the oral cavity. The oral microbial community consisting of more than 700 different bacterial species is considered the second largest microbial community in the human body. Dysbiosis of these microbial communities leads to periodontal diseases such as periodontitis and dental caries. The primary causative agent of dental caries is cariogenic Streptococci, such as Streptococcus mutans. S. mutans form robust biofilms and are difficult to eradicate.

Lactobacillus species are prominent members of the human microbiome and provide beneficial effects by preventing the outgrowth of harmful microbes. Lactobacilli are also the most common probiotic in food. The probiotic effect of Lactobacillus is believed to be due to fermentation, modulation of immune system, and destruction of pathogenic bacteria. Apart from producing organic acids, Lactobacillus also produces bacteriocins and other small effector molecules that inhibit the outgrowth of pathogenic bacteria. Thus, Lactobacillus species can serve as a source for identification of new metabolites with anti-microbial properties.

### Methodology and Results

In this study we have isolated and characterized small organic molecules from Lactobacillus rhamnosus and evaluated their anti-biofilm activities on S. mutans. Cell-free supernatant L. rhamnosus was able to inhibit S. aureus biofilm. The cell-free supernatant was further fractionated into aqueous and organic fractions, and anti-biofilm activities were observed only in organic fraction. The organic fraction was further fractionated using flash chromatography, and the two major fractions obtained were evaluated on S. mutans. The fraction with highest anti-biofilm potential was subjected to FTIR spectra and Mass spectrometry analysis for identification of the active compound. Mass spectrometry and NMR identified the active compound to Cyclo (-L-Leu-L-Pro) and 1mg/ml concentration of the pure compound was sufficient to abolish S. mutans biofilms. Gene expression studies revealed decreased expressions of genes involved in major pathways of biofilm establishment, including two component systems, quorum sensing, EPS formation, and cell surface adhesions in S. mutans.

### Conclusion

We have identified and characterized a metabolite produced by L. rhamnosus effective in the attenuation of the biofilm of S. mutans and can serve as a potential anti-biofilm agent.

### Keywords:

Biofilms, Lactobacillus, Streptococcus mutans

## Development of Novel Sensitisers for use in Antimicrobial Photo and Sono Dynamic Therapy

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### **Background**

Antimicrobial resistance is defined as a change over time, in bacteria, causing them to no longer respond to treatment. This change results in harder to fight infections increasing disease spread and causing severe illness and death. Photodynamic therapy (PDT) is a clinically approved treatment for non-melanoma skin cancer, involving a photosensitiser, a specific wavelength of light and molecular oxygen combining to produce reactive oxygen species (ROS) causing cell death. Sonodynamic therapy (SDT) replaces light with ultrasound (US) overcoming the limited ability of light to penetrate soft tissue. US can penetrate soft tissue up to tens of centimetres allowing for ROS generation in much deeper sections of the tissue. Both PDT and SDT have been shown to exhibit strong antimicrobial activity.

### **Methods**

Novel sensitiser-antimicrobial peptide (AMP) conjugates were synthesised by solid phase peptide synthesis, purified by reverse-phase preparative HPLC. Antimicrobial activity of conjugates investigated by total viable counts. This was undertaken by co-incubating bacteria culture in tryptone soy broth with the conjugate before exposing to light/US. The cultures are then serially diluted and plated on to tryptone soy agar plates and colonies counted and plotted as colony forming units (CFU ml<sup>-1</sup>).

### **Results**

Successful synthesis the conjugate was confirmed using Matrix assisted laser desorption ionisation-time of flight (MALDI-TOF) mass spectrometry showing peaks consistent with the structure of the conjugate. Conjugate without light/US showed significant reduction in CFU ml<sup>-1</sup> against *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Enterococcus faecalis* and an increased antimicrobial effect when combined with light/US using a sub-therapeutic dose of the conjugate.

### **Conclusions**

The use of antimicrobial Photo and Sono dynamic therapy has been observed to exhibit strong antimicrobial effects and may have the potential to improve treatment effectiveness of biofilm infections.

### **Keywords:**

PDT SDT AMP Sensitiser AMR

## Bacteriophage PSE1720 activity in combination with meropenem and evaluation of biofilm reduction in *Pseudomonas aeruginosa* isolated from cystic fibrosis patients

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### Background

*Pseudomonas aeruginosa* is a pathogen responsible of chronic lung infection in cystic fibrosis (CF). Biofilm production impairs the entrance of antibiotics, leading to persistent infection. The aim of this study is to test the activity of phage PSE1720 in combination with meropenem against *P. aeruginosa* CF isolates and to evaluate its ability to inhibit biofilm formation.

### Methods

48 CF *P. aeruginosa* strains were subjected to plaque forming unit (PFU) assay through phage PSE1720 extracted from the hospital wastewater.

Phage kinetics curves were performed using a meropenem concentration equal to MIC value and a phage concentration of  $10^{-7}$  PFU/ml.

Some *P. aeruginosa* strains have been selected for biofilm inhibition study through crystal violet test. Two conditions were examined: i) phage was added after 24h from biofilm formation and ii) after 48h. Another method to assess the reduction/eradication of biofilm was performed involving sonication and colony counting.

### Results

Phage kinetic curves were assessed for susceptible clear and turbid plaques (CP, TP). CP strains showed a growth inhibition ranging from 80% to 92%, showing a good synergism. For TP strains three different pattern were observed: none, moderate or clear synergic effect, with an inhibition rate of 74% compared to 24% for phage alone and 42% for antibiotic alone.

Regarding biofilm for tested strains, one showed no differences in inhibition between the addition of PSE1720 after 24 and 48h (around 4%), while a second showed a 72,57% of inhibition at 24h compared to 40,93% at 48h. For strains producing mucus this approach was not useful and promising results have been obtained by disintegrating biofilm with a Colony Forming Unit  $10^6$  with phage compared to  $10^{12}$  of positive control.

### Conclusions

Both for CP and TP a good synergism was observed combining PSE1720 and meropenem. Results demonstrate that it's necessary to act promptly to prevent the biofilm formation; indeed, the percentage of inhibition decreases when the phage is added after 48h. Because the production of mucus by *P. aeruginosa* strains, which impair the reading of results, the method of disintegration instead of inhibition of biofilm require further investigation.

### Keywords:

phage-antibiotic-synergism, biofilm inhibition, *P. aeruginosa*

## Is there a silver lining for the reduction of biofilm producing wound pathogens?

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### Background

In today's world antibiotic options are becoming more limited due to resistance and the presence of biofilms, and a lack of new antibiotics in the pipeline. Metals, such as silver (Ag) have been long known for their antimicrobial and antibiofilm activity. Silver exhibits antimicrobial activity in different forms including nanoparticles and conjugates, such as silver sulfadiazine. For the treatment of wounds, silver sulfadiazine is approved for direct use on the human body as an ointment. However other forms of silver (e.g., nanocrystalline) are incorporated into wound dressings which release elemental silver into a wound environment. We aim to identify promising compounds to incorporate into an alginate-based hydrogel, which will function as a dermal regeneration scaffold.

### Methods

Compounds of interest were initially screened for their minimum inhibitory concentration (MIC), against 12 strains of bacteria (*E. coli*, *S. aureus*, *P. aeruginosa*, *S. epidermidis*, *A. baumannii*) and the fungus *C. albicans*. Cation-adjusted Mueller-Hinton broth (MHB) and synthetic wound fluid (SWF) were used to determine the MIC and for biofilm inhibition assays. The biofilm inhibitory concentration (BIC) was determined via crystal violet staining for biofilm biomass.

### Results

Silver sulfadiazine has a MIC of 32-256µg/mL in MHB and 4-128µg/mL in SWF across all 12 strains tested, which is as expected. MIC results for silver nanoparticles were inconclusive, as manufacturer concentration (10µg/mL) was a limiting factor; an alternative source, of silver nanoparticles will be required. Biofilm growth curves showed the most promising bacterial strains for BIC assays are *S. aureus* ATCC 6538P, *P. aeruginosa* Pa01, *A. baumannii* NCTC 19606, *S. epidermidis* ATCC 1457. Other bacterial strains and *C. albicans* will be investigated later.

### Conclusions

It was found that different forms of silver can decrease/prevent bacterial growth and that different media can increase/decrease silver efficacy. In the case of silver sulfadiazine, SWF decreases the MIC where for other agents typically it is found to be the opposite. Our next step is to assess the antibiofilm abilities of promising agents within an ex-vivo porcine skin biofilm wound model.

### Keywords:

Biofilm, Silver, Wound-associated infections.

## Probiotic-containing 3D-printed patches for treatment of infected wounds

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### Background

Probiotics have recently received increasing attention in a range of biomedical applications that go behind their traditional field of usage, i.e. the gastrointestinal tract. In particular, probiotic use as live biotherapeutics to treat wound infections refractory to conventional antibiotic treatment is an emerging trend. In this regard, biomaterial-based probiotic encapsulation may represent a promising strategy, not only to protect the vitality of the probiotics but also to facilitate their delivery to the target location. Aim of the present study was to develop 3D-printed patches containing a commercial formulation of live probiotics, and to test their antibiofilm activity in an in vivo-like wound infection model.

### Methods

A blend of 8 different species of probiotics was dissolved in a custom-made bioink, containing sodium alginate as the primary biomaterial, and subjected to extrusion-based 3D printing. Constructs were cross-linked with calcium chloride and their antimicrobial activity was evaluated against pathogenic species in soft agar overlay assays. Finally, the efficacy of the probiotic-loaded patches against biofilms of a wound isolate of *Pseudomonas aeruginosa* was assessed in a collagen wound infection model.

### Result

Through the optimization of several parameters, a bioink with good printability and adequate stability was obtained and successfully loaded with probiotics. When tested in soft agar overlay assays, probiotic-loaded 3D patches displayed a marked time-dependent ability to inhibit the growth of wound isolates, indicating that the metabolites produced by encapsulated probiotics could diffuse through the patch and maintain their antibacterial effect. In the in vivo-like wound infection model the 3D-loaded patches were able to cause more than 2.5 Log reduction (>99%) in the biofilm-associated *P. aeruginosa* load, provided that an appropriate medium for probiotic growth was supplied in the biofilm environment.

### Conclusions

The rise in antibiotic-resistant bacteria, including strains that are resistant to last-resort antibiotics, and the limited ability of antibiotics to eradicate biofilms urge the development of alternative antibacterial therapeutics. Encapsulation of live-bacteria able to contrast the growth of pathogenic strains in appropriate biomaterials seems a promising strategy, allowing a sustained and prolonged release of probiotic-derived bioactive substances at the site of infection, meanwhile reducing potential local and systemic adverse effects.

### Keywords:

Probiotics; infected-wounds; 3D-printing; wound-model; bioink

## Novel synthetic cationic antimicrobial peptides and their potential as antibiofilm agents

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Since the golden era of antibiotics, new drug development has declined, coinciding with the increasing global threat of antimicrobial resistance (AMR) and biofilm-related infections in medical settings. Cationic antimicrobial peptides (AMPs) are intriguing compounds. To address the limitations in the AMP pharmacophore model, two classes of synthetic AMP analogues were developed. 82 novel compounds underwent antibacterial activity tests against various bacterial species to determine their minimum inhibitory concentration values. Initial cytotoxicity was assessed using EC<sub>50</sub> values for human red blood cell lysis. Most candidates showed promising activity and underwent further investigation for the antibacterial mode of action through biosensor-based assays and activity against antibiotic-resistant human clinical isolates. In vivo toxicity was evaluated using a zebrafish model. After the toxicity profile was determined, several compounds were selected for evaluation in vivo in the larval zebrafish infection model. Several lead compounds have shown biofilm inhibition and eradication potential against Gram-positive and Gram-negative biofilm-forming bacteria. Promising compounds were identified to elucidate their mode of action in the antibiofilm using transcriptomics and their activity in vivo. These findings have significant potential in developing new lead compounds, contributing to the ongoing search for antimicrobial solutions to combat AMR and biofilm-related infections.

### **Keywords:**

AMR, AMP, Antimicrobial, Antibiofilm, Zebrafish

## Antimicrobial peptides-loaded PLGA nanoparticles to fight *Pseudomonas aeruginosa*-related infections: unravelling distinct release profiles and effects

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*Pseudomonas aeruginosa* is an opportunistic bacterium commonly involved in persistent infections. Indeed, its well-known ability to form biofilms improves its antimicrobial resistance while countering the host immune system.

The topical application of antimicrobial peptides (AMPs) to treat wound infections is not yet effective as they are sensitive to enzymatic degradation. This work aims to load different AMPs into poly(lactic-co-glycolic acid) (PLGA) nanoparticles (NPs) in order to sustain their antimicrobial effect in the wound environment.

Three AMPs were encapsulated into PLGA NPs by double emulsion: LL37 (+6, 37 residues, 38% hydrophobic), MSI78/pexiganan<sup>®</sup> (+9, 22 residues, 45% hydrophobic) and Dhvar5 (+8, 14 residues, 43% hydrophobic). NPs size and surface charge were characterized by Dynamic Light Scattering, NPs concentration by Nanoparticle Tracking Analysis, while quantification of loaded and released AMP was determined using the fluorescamine assay. Thereafter, AMP-NPs pre-incubated for 72h under stirring at 37°C were assessed for their antimicrobial properties against *P. aeruginosa* in Phosphate Buffer (PBS) and in Muller-Hinton Broth (MHB).

All AMP-NPs were produced at a concentration of 10<sup>11</sup> NPs/mL and showed similar physicochemical properties. Both LL37 and MSI78 were loaded with an efficiency >85%, unlike Dhvar5 with only 50%. After 72h stirring at 37°C, LL37, MSI78 and Dhvar5 reached 6%, 46% and 60% release, respectively. When tested against *P. aeruginosa* in PBS for 2h, LL37-NPs had no antimicrobial effect, while MSI78- and Dhvar5-NPs achieved a total eradication at 4 µg/mL and 1 µg/mL, respectively. In MHB, LL37-NPs had no activity and Dhvar5-NPs lost the antimicrobial effect even at the highest concentrations. Importantly, MSI78-NPs had a concentration-dependent effect, causing a 99% (2 log) bacterial reduction at 32 µg/mL. MSI78-NPs at 4, 8 and 16µg/mL caused no cytotoxic effects on the metabolic activity of human foreskin fibroblasts.

Overall, these findings suggest that the shorter the AMP sequence, the greater the release. MSI78-NPs showed the most effective activity in MHB, as a result of the combined efficient release profile and low MBC (1-4 µg/mL).

### Keywords:

Infected Wounds; Antimicrobial Peptides; Nanoparticles

## Exploring the biofilm breaking potential of artificial sweeteners

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### Background

Antimicrobial resistance (AMR) is recognised by the World Health Organisation (WHO), as one of the most pressing concerns of our time. Over-prescription and incorrect usage of antibiotics have further contributed to the worsening crisis. It is now widely recognised that we are in urgent need of novel therapeutic intervention strategies to tackle the wave of multi-drug resistant infections sweeping through out healthcare systems. This urgent need to identify new compounds with antibiotic properties has prompted scientists to explore new reservoirs to identify potential therapeutics. One reservoir that is emerging with significant therapeutic potential is dietary compounds such as artificial sweeteners. Here, we wanted to explore the antimicrobial and anti-biofilm activity of artificial sweeteners.

### Methods

We perform a range of biofilm assays including polymicrobial and biofilm dispersion assays to identify compounds with anti-biofilm potential. We use RNA-Seq to uncover the mechanism underpinning anti-biofilm activity.

### Results

We show that artificial sweeteners including acesulfame K (ace-K) and saccharin can limit the growth of clinical isolates of *Pseudomonas aeruginosa* and *Acinetobacter baumannii*. Both of these pathogens are notorious biofilm formers and adopting this mode of growth is seen as central to their pathogenic success. We demonstrate that specific artificial sweeteners can inhibit biofilm formation in both of these species, but also that they can disrupt pre-established biofilms. Clinically, many of these pathogens are rarely found in single species biofilms but as part of polymicrobial biofilm communities. We show that both ace-K and saccharin can prevent polymicrobial biofilm formation and disrupt established polymicrobial biofilms comprised of *P. aeruginosa*, *A. baumannii* and *Staphylococcus aureus*. We uncover the specific effects these compounds have on bacterial physiology using live cell imaging and use transcriptomics to confirm that they can disrupt the expression of key genes linked to biofilm formation in *A. baumannii*. Lastly, we demonstrate that these sweeteners can be successfully loaded into hydrogel wound dressings and limit infection progression in an ex vivo porcine burn wound model.

### Conclusions

Our findings demonstrate the influence of artificial sweeteners on pathogen behaviour and uncover their therapeutic potential.

## Synergistic and Antibiofilm activity of short analogues of the marine antimicrobial peptides

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### **Background:**

The rapid emergence of antimicrobial resistance (AMR) has generated an urgent need for new therapeutic strategies. Antimicrobial peptides (AMPs) are considered as promising alternatives to conventional antibiotics due to their rapid membranolytic mode of action against microorganisms in both planktonic and biofilm lifestyles. The aim of this research is to determine the antibiofilm and synergistic potential of synthetic derivatives of marine antimicrobial peptides. Methods: Three biofilm-forming isolates *Pseudomonas aeruginosa*, *Salmonella typhimurium* serovar and *Staphylococcus epidermidis* were used in this study. The minimum inhibitory concentration (MIC) against these isolates, including Gram-positive facultative anaerobe *Clostridium difficile* was determined by the broth microdilution method. In vitro synergy between outer membrane active derivatives and antibiotics was evaluated using the checkerboard methods. The crystal violet staining method was used to investigate the biofilm inhibitory effects of the synthetic analogues. Results: It was found that several synthetic analogues were able to inhibit biofilm formation at sub-MIC levels. Moreover, in checkerboard synergy assays, synthetic peptides showed more pronounced effects on inhibiting static biofilm growth when they were used in combination with other antibiotics. Conclusions: Overall, these results suggest that structural disruption of the bacterial membrane by synthetic derivatives provides a basis for the development of AMPs as adjuvants to treat both planktonic and biofilm infections.

### **Keywords:**

Synergy, Biofilm, Synthetic derivatives, Adjuvants

## Antimicrobial peptide-grafting to improve effectiveness of bacterial wound infection treatment

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The application of antimicrobial peptides (AMPs) to fight biofilm infections and specifically to treat chronic wound infection is not yet effective due to loss of activity in vivo. Our group explores the immobilization of AMPs on polymeric films and nanoparticles as a strategy to reduce AMP aggregation and degradation, improving effectiveness. We use an AMP developed by us, the MSI-78(4-20), a 17-mer short derivative of MSI-78 (commercially known as Pexiganan). MSI-78(4-20) is effective against Gram-positive and Gram-negative bacteria and demonstrated activity against *Pseudomonas aeruginosa* mature biofilms. MSI-78(4-20) was grafted on chitosan thin films by its N- or C- terminus, using a polyethylene glycol heterobifunctional crosslinker (SM(PEG)8). The MSI-78(4-20)-chitosan coating demonstrated bactericidal properties against *Staphylococcus epidermidis* independently of the tethering orientation and an improved performance in the presence of plasma proteins, which mimics conditions that will be encountered in vivo. Therefore, this coating demonstrated a great potential for wound dressing applications.

Moreover, MSI-78(4-20) was grafted to poly(D,L-lactide-co-glycolide)-PEG (PLGA-PEG) nanoparticles (NPs), through a thiol-maleimide Michael addition. Different ratios of PLGA-PEG/PLGA-PEG-Maleimide were tested, and the formulation containing 40% PLGA-PEG-Maleimide displayed the best colloidal properties and the highest AMP content, as evidenced by NPs zeta potential (+8.6±1.8mV) and AMP quantification (326µg/mL). AMP-NPs proved to be as effective as the free AMP with a minimal inhibitory concentration of 8-16µg/mL against *P. aeruginosa* and 32-64µg/mL against *Staphylococcus aureus*. In addition, AMP grafting shortened the time to kill from 1-2h to 15min for *P. aeruginosa* and from 6-8h to 0.5-1h for *S. aureus*. When tested in simulated wound fluid, AMP-NPs showed enhanced antimicrobial activity against *S. aureus* while the opposite effect was observed against *P. aeruginosa*. Importantly, AMP-NPs at a concentration of 16 and 32µg/mL caused no cytotoxic effects on human foreskin fibroblasts with respect to their metabolic activity. Overall, this promising formulation helps unleash the full potential of AMPs for the management of wound infections.

### Keywords:

Antimicrobial peptides, Biomaterials, Wound Infection

## Laser modification of metal surfaces alters biofilm architecture and reduce bacterial biofilm formation

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### Background

Biofilm formation is a basic strategy of bacteria to colonise a surface and may happen on surfaces of any nature whenever bacteria are present. Biofilms are hard to eradicate due to the matrix in which the bacteria reside, consisting of strong, adhesive and adaptive self-produced polymers such as eDNA and functional amyloids. Targeting this biofilm matrix may therefore be a promising strategy to prevent biofilm formation. The aim of this research was to evaluate antibiofilm efficiency of nanotextured stainless steel surfaces.

### Methods

Femtosecond laser pulses generated with Light Conversion Pharos femtosecond Yb-doped solid state laser system were used to modify stainless steel (EI 711) surfaces to introduce either conical spikes or conical grooves. JSM-6700F field emission scanning electron microscope equipped with a JED-2300 energy-dispersive spectrometer (JEOL) was used to analyse the structures and the surface composition. Biofilm assays were performed using *Pseudomonas aeruginosa* PA01 and *Staphylococcus aureus* ATCC25923 standard strains. Leica TCS SPE Confocal system with a coded DMI8 inverted microscope was used to study biofilm structure. Adipose Tissue-derived Stem Cells were used in the eukaryotic cell attachment assay.

### Results

The resulting topography consists of hierarchical nano-microstructures which substantially increase roughness. *P. aeruginosa* PA01 and *S. aureus* ATCC29423 biofilms formed on such nanotextured metal surfaces was considerably modified due to a substantial reduction in amyloid production or due to changes in eDNA surface adhesion, leading to significant reduction in biofilm biomass. Altering the topography of the metal surface, therefore, radically diminishes biofilm development, not by reducing of bacteria on the surface but by altering biofilm architecture. At the same time, growth and colonisation of the surface by a eukaryotic cell line (ADSC) was apparently enhanced, leading to possible further advantages in controlling eukaryotic growth while suppressing prokaryotic contamination.

### Conclusions

Surface nanotexturing can be used to control bacterial fouling in a non-chemical manner, using a low-cost and high-speed method of nanostructures upon laser irradiation.

### Keywords:

nanotexturing, biofilms, antibacterial surfaces

## An Injectable Living Hydrogel with Embedded Probiotics as a Novel Strategy for Combating Multifaceted Pathogen Wound Infections

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### Background

Wound infections pose a significant challenge in healthcare, and traditional antibiotic treatments often result in the development of resistant pathogens. Probiotics have been widely studied for their antimicrobial effects and potential applications in wound treatment as an alternative to antibiotics. However, an effective and efficient carrier system has not been extensively studied so far. In this work, we aim to encapsulate probiotics with proper materials, which can limit the release of probiotics but allow continuous delivery of antibacterial molecules to the infection site, resulting in the alleviation of infection and biofilm formation.

### Methods

We fabricated ProGel, a living hydrogel created by entrapping probiotic *Lactobacillus plantarum* as a therapeutic component within a matrix of adipic acid dihydrazide-modified gelatin, which is crosslinked by functionalized poly(ethylene glycol). With a double-syringe system, ProGel can be easily mixed and applied, conforming swiftly to any wound shape and forming hydrogel in situ. The efficiency of the probiotics encapsulation and the viability of the probiotics inside ProGel were tested using CFU count method and BacTiter-Glo Microbial Cell Viability Assay. The antibacterial properties of the gel are investigated through agar diffusion tests and an ex vivo human skin infection model.

### Results

ProGel demonstrated robust mechanical and self-healing properties owing to the Schiff-base bonds. It sustained more than 80% viability of the entrapped *L. plantarum* while restricting the escape of them from the hydrogel. After a week of storage, more than 70% viability of the entrapped *L. plantarum* was preserved. Importantly, ProGel exhibited broad-spectrum antimicrobial efficacy against pathogens commonly associated with wound infections, i.e., *Pseudomonas aeruginosa* (7 Log reduction), *Staphylococcus aureus* (3-7 Log reduction), and *Candida albicans* (40-70% reduction). Moreover, its cytocompatibility was affirmed through co-culture with human dermal fibroblasts, revealing no cytotoxic effects. The effectiveness of ProGel was further highlighted in more clinically relevant tests on human skin wound models infected with *P. aeruginosa* or *S. aureus*, where it successfully prevented biofilm formation of these pathogens.

### Conclusion

This study showcases an injectable living hydrogel system for the management of complex wound infections.

### Keywords:

Injectable-hydrogels, living-material, probiotics, biofilm, wound-healing

## Iminosugar PDIA as potential novel antibiofilm compound that shows in vivo activity against staphylococcal skin wound infection in mice

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Biofilm-associated infections, still represent a major therapeutic challenge in today's medicine. Iminosaccharides are structural analogues of carbohydrates in which the oxygen atom is replaced by a nitrogen atom. PDIA(beta-1-C-propyl-1,4-dideoxy-1,4-imino-L-arabinitol) is an example of this group of compounds. The present study investigated the effect of PDIA iminosugar under in vivo conditions on skin wound infection caused by *S. aureus* 48, a highly biofilm-forming staphylococcus, in a mouse model.

For the experiments, we used a strong biofilm producing strain of *S. aureus* 48 isolated from a patient with chronic otitis media. The experiment was conducted on male and female BALB/C mice aged between 6 and 10 weeks. Experimental groups established: SA 48: subcutaneous incision with insertion of 50µl 1x10<sup>5</sup> cfu/ml *S. aureus* 48 strain, n=10, SA48 + I: subcutaneous incision with insertion of 50µl 1x10<sup>5</sup> cfu/ml *S. aureus* 48 strain and 500µl of 1mM PDIA iminosugar, n=8. After three days, a wound swabs and tissues were collected for microbiological (qualitative, quantitative determination of bacteria and semi-quantitatively biofilm observation) and histopathological examination (the tissue was analyzed for granulation and neutrophil infiltration).

Microbiological analysis has confirmed that application of PDIA iminosugar significantly inhibited the proliferation of the highly biofilm-forming *S. aureus* 48 strain in mice tissues compared to the group without iminosugar (p=0.0411). In addition, histopathological studies in the group receiving PDIA iminosugar showed reduced tissue granulation (p=3.3908e-05) and accumulation of neutrophils in the tissues (p=0.0011) compared to the group not receiving this iminosugar. The better histopathological parameters in the iminosugar group may indicate a reduced level of biofilm, as we observed semi-quantitatively.

This study provides evidence that PDIA iminosugar can be used as an effective antibiofilm agent for biofilm-associated staphylococcal infections, thus indicating its potential as a treatment option. Additionally, the findings suggest the requirement for further practical investigations, such as exploring the effectiveness of combining treatments with antibiotics or studying carriers matched to iminosugars, to develop efficacious treatment approaches and control infection.

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### **Keywords:**

biofilm, staphylococcus, iminosugar PDIA, mice

## Screening the Chemdiv Antifungal Chemical Library for the Identification of Novel Compounds Targeting Biofilm Formation in *Candida albicans* and *Candida auris*

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### Background

*Candida* spp. are opportunistic yeasts. *Candida albicans* and the emerging pathogen *Candida auris* are prevalent causative agents of candidiasis globally. Both species can form biofilms, contributing to resistance and underscoring the need for novel antifungal therapies.

### Aims

Identify highly active biofilm-formation inhibitors for *C. albicans* and *C. auris*.

### Methods

*C. albicans* SC5314 and *C. auris* strain 0390 were used in this study. A total of 10,000 novel compounds from the Antifungal Chemdiv chemical library were screened using the 96-well microtiter plate model of *Candida* biofilm formation coupled to an XTT-reduction assay in order to identify biofilm formation inhibitors for *C. albicans* and/or *C. auris*. Initial hits presenting a biofilm inhibitory activity > 80% (for *C. albicans*) or > 70% (in the case of *C. auris*) were selected for confirmatory Concentration-dependent (dose-response) experiments. Dose-response experiments, using both metabolic activity and biomass reduction in individual *Candida* biofilms, were performed to reconfirm the activity of initial hits and to determine their potency by calculating the biofilm IC<sub>50</sub> and IC<sub>80</sub> values.

### Results

Using the arbitrary thresholds (80 % and 70 % for *C. albicans* and *C. auris*, respectively), the primary screen revealed 38 compounds with biofilm-inhibitory activity against *C. albicans*, whereas 19 compounds inhibited the formation of *C. auris* biofilms. From the Dose-response experiment, 19 compounds were validated for *C. albicans*, with four compounds presenting an IC<sub>80</sub> lower than two  $\mu$ M for both metabolic activity and biofilm biomass.

### Conclusions

A subset of compounds from the Chemdiv Antifungal Chemical Library was identified as potential inhibitors of biofilm formation for *C. albicans* and/or *C. auris*. While these results are promising, follow-up in vitro and in vivo studies are ongoing to confirm their potential utility for treating candidiasis and potentially other fungal infections.

### Keywords:

biofilm, *Candida* spp, screening, antifungals,

## Antibiofilm compounds from marine natural Arctic and sub-Arctic resources

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### Background

Many approved drugs today are based on knowledge from natural products (ca. 70%) from terrestrial sources and for only 1% of the drugs with natural origin. This indirectly point at marine resources as an untapped reservoir of novel bioactive secondary metabolites. These are important components of the innate defense system in invertebrates in addition to also be a source for potential new drug leads of antimicrobials and anti-biofilm components. The wide repertoire of biological functions that natural compounds/peptides have, and their physical characteristics (small in size, short half-lives, usually non-immunogenic properties, low toxicity) make them ideal for bioprospecting and drug discovery

### Objectives

Identify antibacterial and antibiofilm properties in molecules from marine resources, including marine bacteria, microalgae, plants, and other biological materials like rest raw materials from fisheries.

### Methods

The organisms have been collected from the Arctic or/and sub-Arctic region. Novel bioactive peptides/compounds are isolated, characterised, and explored by traditional bioassay-guided purification of organic extracts in combination with genetic approaches and extensive mechanism of actions studies.

### Results

We have characterized several new groups of antimicrobial peptides and explored their mode of action. In addition, a more extensive screening, performing biosensor tests have revealed mechanisms of action of peptides towards the membrane or integral targets of bacteria.

### Conclusion

Defining the pharmacophores of the molecules by performing SAR studies, in addition to mechanism of action studies, has made it possible to design improved marine natural product mimics that are prosperous as candidates of novel drug leads with specific properties.

### Keywords:

Biofilm, antibacterial, Arctic, Sub-Arctic, marine

## STRATEGIC DRUG REPURPOSING FOR BIOFILM ASSOCIATED BACTERIAL VAGINOSIS

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### Background incl. aims

Bacterial vaginosis (BV) is a common disorder characterised by an imbalance of the vaginal microbiome, leading to a reduction in beneficial lactobacilli and the formation of polymicrobial anaerobic biofilms. Despite its high prevalence, treatment options are limited, with reoccurrence rates of up to 40% observed with the most commonly used antibiotics metronidazole and clindamycin. This study therefore aimed to investigate repurposing of existing anti-infectives for antimicrobial activity against *Gardnerella vaginalis* and associated multi-species biofilms representative of BV.

### Methods

Two drug repurposing libraries; the global health priority box (n=240 compounds) and the pandemic response box (n=400 compounds) from the medicines for malaria venture (MMV) and drugs for neglected disease initiatives were initially screened for antimicrobial activity against planktonic *G. vaginalis* ATCC 14018. Hits from the initial screen (n=640) were then streamlined based on activity against commensal *Lactobacillus crispatus* and cytotoxicity against VK2 vaginal epithelial cells. Successful hits based on prior screens were then screened for anti-biofilm activity against mono-species *G. vaginalis* biofilms, before further qPCR based testing against a polymicrobial BV biofilm model comprising *G. vaginalis*, *Fannyhessea vaginae*, *Mobiluncus curtisii* and *Prevotella bivia*.

### Results

From 640 compounds, we demonstrated that 28 compounds were able to significantly inhibit (>90%) growth of *G. vaginalis*. Of these hits, 18 compounds were removed from analysis as they demonstrated antimicrobial activity against *L. crispatus* and/or cytotoxicity against VK2 cells, leaving 10 compounds for biofilm analysis. Of the 10 compounds, MMV1634360, an anti-cancer drug, demonstrated robust biofilm activity at a range of concentrations and was able to significantly reduce the bioburden and composition of the multispecies BV biofilm.

### Conclusions

In this work, we have identified existing and approved anti-infective drugs as suitable candidates for repurposing in bacterial vaginosis. These compounds showed significant and selective inhibition of *G. vaginalis* growth when compared with *L. crispatus* and minimal cytotoxic effects. Importantly, one compound, MMV1634360 showed efficacy against *G. vaginalis* mono- and multi-species biofilms. Current work is ongoing, investigating these compounds against antibiotic-resistant clinical isolates and using a transcriptomics based approach to identify the mechanism of action of this compound.

## Characterisation and disruption of extracellular polymeric substances in in vitro oral biofilms

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### **Background and Aim**

Biofilms occur when microorganisms aggregate on surfaces and produce extracellular polymeric substances (EPS) in which the microbial cells are embedded. Biofilms are involved in a wide range of diseases and exhibit increased resistance to antimicrobial agents due to numerous and complex mechanisms, several of which depend upon EPS. This work aimed to characterise the EPS of biofilms and then evaluate strategies to disrupt the EPS and render the biofilm cells more susceptible to antimicrobial therapies.

### **Methodology**

In vitro biofilms were grown using single and mixed microbial species (*Candida albicans*, *Streptococcus mutans*, *Streptococcus Salivarius*, *Streptococcus gordonii*, *Streptococcus sanguinis*) and whole saliva as inocula. The biofilms were developed on polycarbonate coupons under batch or flow-through culture, with different culture media, incubation time and periodic exposure to drying. The EPS of the resulting biofilms was assessed with fluorescently tagged molecular probes and confocal laser scanning microscopy (CLSM), followed by evaluation of enzyme treatment targeting the EPS.

### **Results**

CLSM revealed EPS production was dependent on growth conditions. Intermittent drying or extended incubation did not alter EPS production, which was primarily detected near cells and in limited quantities. Importantly, addition of dextran and sucrose to the culture medium increased EPS in *S. mutans* biofilms as seen by incorporation of fluorescently labelled dextran into the biofilm matrix. Dextranase subsequently disrupted the glucan matrix of these *S. mutans* biofilms.

### **Conclusion**

Studies showed that in vitro biofilms had low levels of EPS that were detectable using fluorescent probes and CLSM. This was the case for most assessed growth variables. However, medium supplementation with dextran and sucrose for *S. mutans* biofilms yielded high levels of EPS. CLSM revealed that dextranase successfully disrupts the glucan matrix. Further work will assess potential synergistic effects of enzymatic treatment with conventional antimicrobials for biofilm disruption.

### **Keywords:**

Biofilm Matrix Disruption CLSM EPS

## Effect of the BDNG001 dendrimer on Candida biofilm formation and gene expression

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Millions of people suffer from fungal infections around the world and among the microorganisms responsible is the genus *Candida*. These pathogens are a major source of device-associated infections because of their capacity to form biofilms, microbial communities that are difficult to eliminate and resistant to antifungals. Therefore, finding compounds capable of eradicating them and understanding how they are affected is essential. Dendritic systems are a promising alternative in the field of biomedicine. These systems have a well-defined and monodisperse structure, and multivalence, among other properties, that give them the ability to act as antimicrobial drugs. The aims of this study were to evaluate the activity of the BDNG001 dendrimer on *Candida* spp. biofilms and to determine its effect on gene expression.

The Minimum Biofilm Inhibition Concentration (MBIC) and the Minimum Fungal Biofilm Concentration (MFCB) of the BDNG001 dendrimer were determined against *C. albicans* and *C. glabrata*. For the study of gene expression, *Candida* spp. was grown for 48 h. The strains were treated at sublethal concentrations (sub-MBIC). Then, biofilms were collected, RNA was extracted, and qPCR performed. Different genes related to biofilm and hyphal formation were evaluated.

Data from this study showed an MBIC value for the BDNG001 dendrimer of 16 mg/L against *C. albicans* and 8 mg/L against *C. glabrata* (non-cytotoxic concentrations). An alteration in the expression of some genes involved in biofilm formation was observed for both *Candida* species in the presence of the dendrimer at sublethal concentrations. In the literature, some studies have shown that sub-MBIC effect of antimicrobials can induce or decrease biofilm formation, down- or upregulate gene expression. Therefore, the effect of these sublethal concentrations remains controversial. In addition, *ERG11* and *CDR1* genes were also affected.

We can conclude that the BDNG001 dendrimer is an interesting anti-biofilm candidate for further in vivo studies and its presence interferes *Candida* gene expression.

### **Keywords:**

biofilm, *Candida*, dendrimer, gene expression

## In vitro activity of a carbosilane dendritic system against *Candida tropicalis* biofilms: activity, mode of action and resistance induction study

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*Candida* spp. is an opportunistic fungal pathogen responsible for serious infections, making it necessary to improve their prevention and treatment. *Candida tropicalis* is included in the high-priority group according to WHO. The treatment of these infections is challenging due to their resistance to commercial antifungals and their ability to form biofilms. Biofilms are problematic because they easily develop on medical devices, making them a major concern in hospitals. In addition, these communities are extremely resistant, making it difficult to eradicate *Candida* infections and considering them a threat to antimicrobial resistance. Therefore, considering the difficulty to treat *Candida* biofilms, it is necessary to focus on finding new preventive measures and treatments against them. In this regard, carbosilane dendritic systems are promising therapeutic agents. The aims of this study were to evaluate the in vitro activity of a cationic dendrimer (BD132) inhibiting biofilm formation and eradicating *C. tropicalis* cells, understand more about its effect and analyse if the compound induces resistance in *Candida* cells.

First, the microdilutions method on a 96-well plate and the drop plate method were used to test the in vitro capacity to inhibit biofilm formation. We also employed these methods to analyse the synergistic effect of the dendritic compound with some commercial antifungals. Second, we evaluated if the compound induces resistance in *Candida* cells by adding compound every day for 15 days. Additionally, we studied if the compound was stable during this period. Finally, the compound effect on *Candida* cells was analyzed by scanning electron microscopy (SEM) and flow cytometry. The results showed that the compound was effective against biofilms at non-cytotoxic concentrations. Moreover, the compound showed a synergistic effect when combined with both, AgNO<sub>3</sub>, and Amphotericin B. Furthermore, the dendrimer did not induce resistances in *Candida* cells and it was stable for more than 15 days. Cytometry and SEM studies revealed morphology and membrane alteration.

Based on the results, the dendrimer seems to be an attractive therapeutic agent, being interesting to consider further investigation to learn more about its mechanism of action and test its activity and toxicity in animal models.

### **Keywords:**

biofilm, *Candida*, dendrimer, resistance, synergy

## Normobaric oxygen therapy augments killing of slow growing *Pseudomonas aeruginosa* by quinolones

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### Background

Chronic pulmonary infections harboring bacteria with low susceptibility due to slow growth or limited aerobic respiration are considered a major problem of modern medicine. New strategies are needed to improve antibiotic treatment of slow-growing bacteria such as chronic *Pseudomonas aeruginosa* (Pa) infection. In the infected part of cystic fibrosis (CF) lungs, the consumption of oxygen (O<sub>2</sub>) by the surrounding neutrophils results in large zones of oxygen (O<sub>2</sub>) depletion, whereby bacterial aerobic respiration is prevented. Restriction of O<sub>2</sub> may reduce the uptake of antibiotics, down-regulate antibiotic targets and inhibit induction of bactericidal amounts of reactive O<sub>2</sub> species. We hypothesize that by enhancing the aerobic physiology of the bacteria using adjuvant O<sub>2</sub> supplied by normobaric O<sub>2</sub> treatment (NBOT) (100% O<sub>2</sub>, 1 ATA, 90 min), the bacteria can be sensitized to quinolones in doses that can be attained clinically through systemic or inhaled therapy.

### Methods

The effect of NBOT on quinolone treatment was tested using Pa embedded in agarose. Re-oxygenation of both in vitro growing Pa and sputum samples from persons with CF (pwCF) was confirmed by O<sub>2</sub> profiling.

### Results

NBOT significantly enhanced the killing by ciprofloxacin and levofloxacin of Pa in vitro by 2 to 4 log units using 0.25 to 2 mg/L and 1 to 16 mg/L respectively. The ability to re-oxygenate anoxic zones by NBOT was demonstrated in freshly expectorated sputum samples from pwCF with a build-up of O<sub>2</sub> in layers below the surface. Activation of aerobic respiration in sputum was indicated by the increased O<sub>2</sub> consumption during NBOT.

### Conclusion

This proof-of-principle study demonstrates that reoxygenation of the infectious microenvironment by NBOT can significantly enhance the susceptibility of Pa to quinolones. Direct measurement of O<sub>2</sub> consumption in sputum samples can be considered for obtaining proof-of-concept for the advantageous use of adjuvant intermittent NBOT in adult pwCF.

### Keywords:

Normobaric oxygen, Quinolones, *P. aeruginosa*

## Disrupt and deliver: ultrasound-mediated lipid-based drug delivery to tackle biofilm infections

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Bacterial cells can grow and survive in a coordinated manner within three-dimensional structures known as biofilms. In biofilm, bacteria are up to 1000 times more resistant to antibiotics than when in planktonic form. Biofilms being prevalent in many infections are of clinical importance, however, there are no approved anti-biofilm treatments available.

Nanoscale lipid-shelled vesicles, known as liposomes, have been shown to be effective at delivering drugs to bacterial cells. Unfortunately, they are only efficient at targeting cells at the biofilm surface, meaning cells within the biofilm can persist. Microbubbles are gas-filled particles that can be activated by ultrasound to physically destabilise biofilms and disperse cells, allowing access into the deeper layers.

The aim of this research is to conjugate antibiotic-loaded liposomes to microbubbles, creating ultrasound-sensitive particles. These particles can both disrupt bacterial biofilms upon ultrasound exposure and deliver drugs to dispersed and embedded bacteria through uptake of liposomes. Cationic and fusogenic liposomes encapsulating vancomycin were made using the thin-film hydration method followed by manual extrusion. Liposome size and zeta potential were measured using dynamic light scattering (DLS) and monitored over 7 days to assess storage stability. Ultrafiltration was used to remove non-encapsulated vancomycin and encapsulation efficiency was measured using UV spectroscopy. The standard broth microdilution method was used to determine minimum inhibitory concentrations (MICs) of free vancomycin and both liposome formulations against 4 bacterial strains.

Cationic and fusogenic liposomes were formulated with average diameters of 126 nm and 102 nm and zeta potentials of +25 mV and -40 mV, respectively. These properties of both formulations remained consistent over a period of 7 days after storage at 4°C. Vancomycin-encapsulated fusogenic liposomes (30-40% encapsulation efficiency) gave the lowest MIC (<500 µg/mL) compared to cationic liposomes (~1200 µg/mL) and free vancomycin (2000 µg/mL) when tested against *P. aeruginosa*. Liposomes with good storage stability and high encapsulation efficiency were produced.

Encapsulating vancomycin within fusogenic liposomes likely enhances its antibacterial activity, even against Gram-negative bacteria, and could provide an avenue for enhanced drug delivery into bacterial cells. This provides a good basis for encapsulation of other antibiotics, such as polymyxins, that present major toxicity concerns.

### **Keywords:**

liposomes, microbubbles, ultrasound

## In vitro evaluation of the antibacterial activity of five daily-use mouthrinses with different active ingredient concentrations

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### Background and aims

Chlorhexidine (CHX) and Cetylpyridinium Chloride (CPC) are antiseptic molecules formulated in mouthrinses for daily use. In the present study we have compared the antimicrobial activity on multispecies oral biofilms of five mouthrinses containing CHX and/or CPC (A: 0.12% CHX and 0.05% CPC; B: 0.2% CHX and CPC: Concentration unknown; C: 0.2% CHX; D: 0.2% CHX; E: 0.2% CHX).

### Methods

Oral biofilms formed by *S.oralis*, *A. naeslundii*, *V. parvula*, *F. nucleatum*, *A. actinomycetemcomitans* and *P. gingivalis* grown on hydroxyapatite discs for 96 hours at 37°C under anaerobic conditions. The mouthwash treatment was administered for 2 minutes. The biofilms were then mechanically disrupted for 5 minutes in 1 ml of phosphate-buffered saline. Survival was calculated by counting viable colonies grown on blood agar and Dentaid-1 culture plates. Kruskal-Wallis and Dunn's test were used for all comparisons.

### Results

Significant differences were observed in the anti-biofilm efficacy of the five products tested (p-value < 0.05). Mouthwash A caused a significantly higher absolute mortality than the other formulations (p-value AvsB < 0.001; p-value AvsC < 0.001; p-value AvsD < 0.001; p-value AvsE < 0.001). Mouthwash B caused a significantly higher absolute mortality than formulation E (p-value BvsE 0.029). The percent of mortality was also significantly higher for mouthwash A (A: 78.57%, B: 39.50%, C: 25.28%; D: 20.32% E: 16.15%). No significant differences were observed when comparing the antimicrobial activity of E and C; E and D; C and D; C and B; D and B (p-value EvsC: 0.17; p-value EvsD: 0.918; p-value CvsD: 0.627; p-value CvsB: 0.863 p-value DvsB: 0.178).

### Conclusion

Mouthrinse A showed the greatest anti-biofilm power. The mouthrinses with CHX and CPC in their formulation showed greater antimicrobial capacity.

### Keywords:

mouthwash, CHX, CPC, antimicrobial, antiseptic

## Synergistic effect of p-bromodomiphen bromide with conventional antifungals against *C. auris*

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### Background

*Candida auris* is an emerging opportunistic yeast which can develop biofilms allowing its persistence on environmental surfaces and human niches. Most *C. auris* strains (90% of isolates) display fluconazole (FLZ) resistance, 30% resistance to amphotericin B (AmB) and 5% to echinocandins. The limited therapeutic options have prompted researchers to explore drug combinations or potential alternatives for *C. auris* treatment. Quaternary ammonium compounds (QACs) are known for their biocidal activity. Domiphen bromide (D-Br) has been shown to potentiate triazole activity against *C. albicans*. Its derivative, p-bromodomiphen bromide (B-Br), has been recently enlisted as a new, potent, and fast-acting QAC. In this study, we tested B-Br and D-Br against *C. auris*, in both planktonic and sessile cells, in combination with FLZ or AmB.

### Methods

Two clinical strains of *C. auris* with different antimicrobial susceptibility patterns (CaS, resistant to FLZ; CaR, resistant to FLZ and AmB) were used. FLZ (512.0-0.25 µg/mL), AmB (8.0-0.03 µg/mL), and B-Br (64-0.03 µg/mL) were used alone or in combination. Broth microdilution and XTT assays were used for planktonic and sessile antimicrobial susceptibility testing, respectively. Checkerboard tests were performed to study the interaction between QAC and conventional antifungals. To assess the cytotoxicity of B-Br, MTT assay was performed on HaCat and Hep-2 cell lines.

### Results

Fluconazole planktonic MICs were >256.0 µg/mL and 128.0 µg/mL, respectively for CaR and CaS, whereas for AmB MICs were 2.0 and 1.0 µg/mL. For B-Br, MICs were 4 µg/mL and 2 µg/mL for CaR and CaS, respectively. When testing *C. auris* biofilms, fluconazole MICs progressively increased from adhesion to mature biofilm up to >512.0 µg/mL, whereas for AmB MIC were stable. The fractional inhibitory concentration index (FICI) indicated a synergistic effect of B-Br with both FLZ and AmB against CaS and CaR biofilms (FICI-AmB/B-Br=0.37 and FICI-FLZ/B-Br =0.5 for both sessile isolates).

### Conclusions

Br synergize with FLZ and AmB resulting in a stronger inhibitory activity also against *C. auris* biofilms, without exerting toxicity. In combination, the concentration of the FLZ and AmB fell within the range of concentrations considered effective in clinical practice.

### Keywords:

*C.auris*, Synergy, QAC, Resistance, Biofilm

## Exciting new hydrogel coating to prevent biofilm formation on medical devices with no kill effect

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### Background and aims

Biofilm formation on medical devices is the major cause of healthcare-associated infections, e.g., Catheter Associated Urinary Tract Infection (CAUTI). There is no effective, long-lasting coating available on the market that prevents biofilm on medical devices. Existing coatings are antibacterial and release antibiotics or silver, depleting after a short time and promoting antimicrobial resistance. Bacteria and fungi adhere to virtually all natural and synthetic surfaces, as adhesion is crucial for their survival. The characteristics of the pathogens, environmental factors and the physical-chemical parameters of the surface all affect the adhesion which in turn regulates the formation of biofilm. Depending on the mode of surface adhesion, microbes tailor the synthesis and excretion of extracellular-polymeric-substances (EPS). Colonizing microbes that lack the shield of the EPS, are easier targets for the immune system and more susceptible to treatments. Our strategy to fight back against biofilm is a unique anti-fouling coating that prevents biofilm formation (EPS) providing long-term efficacy thanks to a permanent bonding and a no-kill strategy.

### Methods

We have developed a novel process for coating medical device polymer materials with our proprietary anti-fouling and anti-biofilm coating. The process includes a unique UV-induced polymerization method for grafting a polyacrylic acid polymer to a device surface before coupling of our proprietary ligand through dipping. This new coating protocol is suitable for a wide range of materials; it is inexpensive, easy to scale up and uses readily available non-toxic chemicals. CytaCoat's unique polymer grafting process provides a permanent, low-friction hydrogel coating.

### Results and conclusions

In-house and external laboratory tests demonstrated that CytaCoat-coated silicone urinary catheters were smooth, superhydrophilic and did not leak any substances. Biofilm fluorescent staining of the catheters from 7-day long cultures of nine different strains of common uropathogens, including *Candida albicans*, showed substantially lower EPS-formation in artificial urine. We have developed a new low-friction superhydrophilic coating for urinary catheters that effectively prevents biofilm formation of a broad spectrum of pathogens and is therefore a promising technology to prevent healthcare-associated infections and antimicrobial resistance. Further applications of the coating are in development.

### Keywords:

EPS, antifouling, superhydrophilic, urinary catheter

## Hyaluronic acid hydrogel reservoirs: A novel approach with antimicrobial peptides to combat biofilms

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The increasing occurrence of chronic wound infections and the rise of antibiotic-resistant bacteria present a serious global challenge. The effectiveness of traditional antibiotics is often compromised by biofilms, making these treatments less effective. This urgency has driven the development of alternative strategies to combat biofilm-associated chronic wound infections. Our research evaluates the effectiveness of antimicrobial peptides against established biofilms and explores the use of Hyaluronic Acid (HA)-based Hydrogels, infused with these peptides, for improved treatment outcomes.

This study investigates the effectiveness of antimicrobial peptides Poly(arginine)<sub>30</sub> (PAR30) and Poly- $\epsilon$ -Lysine ( $\epsilon$ -PLL), both in solution and incorporated in hydrogels, against preformed biofilms of *Staphylococcus aureus* and *Pseudomonas aeruginosa*. The biofilms were formed under static conditions using the test piece holder system or microtiter plate assay, and under dynamic conditions with the CDC biofilm reactor, loaded with materials like titanium, silicon, and PEHD to simulate real-world environments.

Our findings indicate that  $\epsilon$ -PLL and PAR30 have minimum inhibitory concentrations ranging from 16 to 32  $\mu\text{g}/\text{mL}$  against both bacterial strains. The results highlight the strong antibiofilm properties of these peptides compared to traditional antibiotics and cathelicidin LL-37, at doses 5 to 10 times the MIC. Treatment of static biofilms grown for 5- and 24-hour with HA-based Hydrogels containing these antimicrobial peptides showed significant biomass reduction (up to 97%) across all tested strains, demonstrating their potent antibiofilm efficacy. HA-based Hydrogels with  $\epsilon$ -PLL were also highly effective in eradicating *P. aeruginosa* biofilms grown in dynamic conditions ( $> 5$ -log reduction), while showing low cytotoxicity to human fibroblasts. Furthermore, we explored the compatibility of HA-based Hydrogel with various medical materials, achieving up to 95.75% reduction in pre-formed static *P. aeruginosa* biofilm biomass.

In conclusion,  $\epsilon$ -PLL and PAR30 are promising as antibiotic alternatives, especially when incorporated into hydrogels that could be applied to medical devices. Our results underscore the potential of these HA-based Hydrogels in eradicating pre-formed biofilms.

### **Keywords:**

Antimicrobial peptides, Biofilm, medical device

## EFFICACY OF NITROFURAN AND INDOLE DERIVATIVES AGAINST HISTOPLASMA CAPSULATUM BIOFILM AND PHARMACOLOGICAL SAFETY IN THREE-DIMENSIONAL LUNG CELLS CULTURES AND IN VIVO

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Histoplasmosis is a systemic mycosis caused by the dimorphic fungus *Histoplasma capsulatum* of cosmopolitan distribution. In 2022, the World Health Organization (WHO) classified this fungus as a high priority. Histoplasmosis therapy is long and complex, as the antifungal agents used cause many toxic effects, relapses, and refractory cases. Nitrofurans and indole derivatives have already demonstrated efficacy against fungi. Additionally, there is evidence that the fungus *H. capsulatum* can form biofilms, which are sessile communities that allow microorganisms to survive in hostile environments. This study aimed to assess the efficacy of nitrofurans against biofilms formed by *H. capsulatum*. Additionally, the toxicity of these derivatives was evaluated in alternative models such as *Caenorhabditis elegans*, *Galleria mellonella*, and Zebrafish, as well as in three-dimensional (3D) lung cells cultures. The metabolic activity of biofilms was evaluated using the tetrazole salt reduction (XTT) method after treatment. The study utilized scanning electron microscopy (SEM) and confocal microscopy to visualize damage to mature biofilms. The cytotoxicity index (CC50) was calculated using GraphPad Prism 5.0 software, and the animal survival curve was plotted. The compounds were highly effective against both early-stage and mature biofilms at concentrations equal to or twice those required to kill planktonic fungal cells (7.81 at 31.25 µg/mL). The micrographs show a reduction in biofilm metabolic activity, thickness, and extracellular matrix. The compounds exhibited low toxicity in 3D cultures, with a CC50 of 13.22 µg/mL at concentrations of ≥ 250 µg/mL. Furthermore, low toxicity or no toxicity was observed in alternative models of *C. elegans*, *G. mellonella*, and zebrafish, even at high concentrations. These results are important in the quest for new potent alternatives, especially against fungal biofilms.

DEVELOPMENT AGENCY: CAPES, CNPq and FAPESP - Process 2020/15586-4; 2022/15826-0.

### Keywords:

antifungal, biofilm, 3D-culture, alternative animals

## Internalization of ethanol in porous silica nanoparticles as an aim to improve catheter lock therapy.

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### Background

Combining systemic and lock antibiotic therapies is recommended for the conservative management of long-term catheter-related bloodstream infections (C-RBSI). Ethanol (ET) is being used as an alternative to antibiotics because of its advantage of not being associated with resistance. However, we observed that sessile cells treated with ET and re-exposed to culture medium were able to regrow. Therefore, it is necessary to optimize ET penetration with innovative strategies. We aimed to design and prepare ET-based microporous silica nanoparticles (NP) to release it at a growth rate enough to penetrate through biofilm and extracellular matrix.

### Methods

We added tetraethyl orthosilicate (TEOS) as a surfactant to an aqueous solution of: sterile water, 96%ET, cetyltrimethylammonium chloride (CTAC), and diethanolamine (DEA) at 40°C, followed by a calcination step of 6h at 550°C to eliminate the surfactant. Characterization of NP was carried out by transmission electron microscopy (TEM), scanning electron microscopy (SEM), dynamics light scattering (DLS), zeta-potential, and nitrogen absorption. NP impregnation with 96%ET was performed by a sonication bath and ET release profile was analyzed using gas chromatography-mass spectrometry (GC-MS).

### Results

We obtained homogeneous microporous silica NP of  $77.3 \pm 3.06$  nm of average diameter (fig 1a) and  $-22.3 \pm 1$  mV of potential Z, which were loaded with 96% ET into pores of 1.8 cm<sup>3</sup>/g volume. The release profile of ET obtained with GC-MS is detailed in fig 1b in which 96% ET was released after 1h.

### Conclusions

Our is the first experimental study in which we have successfully design and elaborate microporous silica NP with a hydrodynamic size below 100 nm, capable of releasing ET to be used as an improved ET based-catheter lock solution against microbial biofilms and with a hydrodynamic size below 100 nm to penetrate into biofilm matrix channels. Future studies are needed to demonstrate that encapsulated ET significantly reduces microorganisms' regrowth compared to conventional ET lock solution.

"THESE PRELIMINARY DATA WILL BE SUBMITTED FOR PATENTING IN THE COMING MONTHS, SO PLEASE DON'T DISSEMINATE OR DISCLOSE THE INFORMATION CONTAINED IN THIS WORK. IN CASE OF ACCEPTANCE BY THE ECCMID REGULATORY COMMITTEE, WE WILL LET YOU KNOW IF THE PATENT HAS BEEN PROCESSED. THANK YOU VERYMUCH"

**Keywords:**

microporous silica; nanoparticles; biofilm; ethanol.

## Microbubble Mediated Mechanical Removal Of Oral Biofilms In-Vitro

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### Introduction/background

The oral microbiota comprises of both commensal and pathogenic bacteria, that live attached to tooth and soft tissue surfaces forming complex and long-lasting biofilms. Despite some promising anti-plaque methods being developed, the lack of truly efficacious therapeutic options constitutes an unmet need for effective treatment of oral infections. Given the high human and economic burden of oral biofilm-related infectious pathologies, alternative methods for dental plaque removal are a research area of growing interest. In this work, we hypothesise that ultrasound induced cavitation of gas microbubbles can be employed as an effective modality for mechanical removal of oral biofilms from the tooth surface.

### Methods

A single species biofilm composed of *S. mutans* was grown on a hydroxyapatite (HA) disc, which was used as a tooth mimicking surface. Bacteria were cultured on brain heart infusion agar and the biofilm was grown statically for 24h.

The microbubble shell constituents were dipalmitoylphosphatidylcholine and polyoxyethylene-(40) stearate in a 9:1 molar ratio, while the gas core consisted of perfluorobutane.

The treatment involved submerging the HA disc in ~2 mL of microbubble suspension. The disc was placed at the focus of a high-intensity focused ultrasound transducer. Ultrasound (US) parameters used were: driving frequency of 0.5MHz, 1000 cycles per burst, pulse repetition frequency (PRF) of 5Hz, duty cycle (DC) of 1%, and peak negative pressure (pnp) between 0.2 and 2.0MPa. The total treatment time was of 60 seconds.

### Results

Results show a positive correlation between the microbubble cavitation activity (i.e., a measure of ultrasound responsiveness) at the biofilm interface and the removal of bacteria from the surface. The effect of cavitation was either restricted to the focal region of the US field or affected a much larger area, depending on the peak negative pressure applied. These findings suggest that US-induced microbubble cavitation could improve the removal of plaque in hard-to-reach crevices in the oral cavity.

### Conclusions

We demonstrated that exposing gas microbubbles to ultrasound of low duty cycle and relatively low acoustic pressure, can mechanically damage biofilms formed on a tooth mimicking hard surface. This could be further developed as an anti-plaque treatment in biofilm-related oral infections.

### Keywords:

Ultrasound, Cavitation, Oral-biofilm, Plaque, Cavities

## Interactions between phages and *P. aeruginosa* biofilms

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*Pseudomonas aeruginosa* is an important pathogen causing chronic infections in the lungs of patients with cystic fibrosis (CF) and non-CF bronchiectasis. Maintenance of the patients' lung function requires prolonged antibiotic therapy which promote development of antimicrobial resistance and therefore alternative therapeutic approaches are required.

In this project, we investigate the effect of phage therapy on biofilms, and specifically how its efficiency is influenced by the genetic background of the *P. aeruginosa* strains.

Seven different strains were examined: two reference strains (PA14, PAO1), the Pf4 prophage-deficient mutant PAO1ΔPf4, its wild-type PAO1wt, and three clinical CF isolates. For quantification of the phage effects on biofilm formation, one day old biofilms were grown on glass beads and treated with 10<sup>8</sup> PFU/mL of the lytic bacteriophage NP3. Further, phage effects on biofilms of different ages (24, 48 and 72 h) as well as multiple phage treatments were also investigated for PA14, PAO1, PAO1ΔPf4 and PAO1wt.

The effects of NP3 on *P. aeruginosa* biofilm formation were assessed by crystal violet staining and quantification of biofilm-associated culturable bacteria and viable phages after washing and sonicating the biofilms. Measurements of the oxygen consumption by optical fluorescence-based oxygen respirometry were also performed.

In general, a reduction in the biomass of the *P. aeruginosa* biofilms after NP3 treatment was observed for all *P. aeruginosa* strains, except for PAO1 and PAO1ΔPf4 in which a biomass increase was observed after treatment with NP3. A two-three log reduction in CFU/mL was observed for all strains. The number of phages remained at 10<sup>8</sup> PFU/mL in all biofilms.

Treatment of biofilms of different ages with NP3 showed the highest effect in the youngest biofilms. The biomass of biofilms older than 24-hours was unaffected, despite a decrease in cell viable counts and bacterial metabolic activity. Multiple phage treatments of PAO1 and PA14 biofilms resulted in decreased effect of the phage exposure after the first treatment.

To conclude, the effect of phage treatment on *P. aeruginosa* biofilms is strain-dependent. However, in all cases single treatment of younger biofilms was most effective, and the anti-biofilm effect of phage treatment decreased after multiple exposures.

### **Keywords:**

Bacteriophage, Biofilm, *P. aeruginosa*

## Sodium bicarbonate removes dental plaque by disrupting its mechanical stability

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### Background

Dental biofilms, also known as dental plaque, constitute intricate microbial communities attached to tooth surfaces and restorations. These biofilms generate a matrix of polysaccharides, eDNA and lipoteichoic acid, providing mechanical stability and protection from antimicrobials. The acids from biofilm bacteria can lead to tooth enamel dissolution, causing cavities, and contribute to gum disease (periodontitis) by inducing gum inflammation. Prevention and treatment of dental biofilms involve a multifaceted approach, including mechanical removal through brushing and flossing, antimicrobial agents like mouthwash and toothpaste, and professional cleaning by dentists. Sodium bicarbonate in certain dentifrices has been shown clinically to remove and reduce dental plaque biofilm even in hard-to-reach areas of the mouth. This study aims to investigate the underpinning mechanism of action of sodium bicarbonate treatment on the mechanical properties of oral biofilms.

### Methods

Particle tracking microrheology was used to quantify the viscoelastic properties of *Streptococcus mutans* biofilms grown under shear conditions. By measuring the changes in viscoelastic properties of biofilm post-treatment, the influence of sodium bicarbonate on the mechanical properties of biofilms can be investigated. Confocal imaging was used to visualize the structural changes in biofilms.

### Results

Under shear conditions, *S. mutans* developed a robust biofilm, exhibiting viscoelastic properties with varying stiffness throughout its layers. The layer closest to the substratum was found to be firmer than the upper layers, with the elastic modulus ranging from about 70 Pa at the bottom to about 1 Pa at the top surfaces. Treatment with 67% sodium bicarbonate effectively removed the softer upper layers, resulting in a 40% decrease in biovolume. However, the lower biofilm layers were unaffected by the sodium bicarbonate resulting in an increase in the measured average stiffness with an elastic modulus of about 70 Pa. Subsequent retreatment further softened the remnant biofilm and reduced the biovolume by 80%.

### Conclusions

This suggests that 67% sodium bicarbonate removes *S. mutans* biofilms by softening the matrix and disrupting the mechanical stability and structural integrity of biofilms.

### Keywords:

Oral biofilm, microrheology, Sodium bicarbonate,

## Identification of an anti-biofilm squalamine derivative against *Pseudomonas aeruginosa*

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*Pseudomonas aeruginosa* is an opportunistic pathogen responsible for numerous acute and chronic infections in humans. It is known for its resistance to many antibiotics, linked to its ability to form biofilms. It is therefore urgent to find alternative strategies to combat this bacterium. Squalamine, known for its multiple activities (anti-bacterial, anti-fungal, anti cancer, etc.) is an aminosterol extracted from the shark *Squalus acanthias*. Although this compound offers interesting properties, its synthesis remains long, costly and complicated. Derivatives of this molecule were therefore synthesized and screened to identify new compounds with anti-bacterial/anti biofilm activities to fight *Pseudomonas aeruginosa*. The minimum inhibitory concentration of the compounds was determined according to EUCAST conditions. The effect of compounds at different sub-inhibitory concentrations was screened on growth, pyocyanin production, biofilm formation and dispersion (crystal violet method). Compounds affecting these phenotypes by at least 50% were selected. A bacterial biofilm, formed for 24 h, was then exposed to 2 compounds for 2 h, then observed under a confocal laser scanning microscope (CLSM) after biomass labelling with SYTO9. Images were analyzed by COMSTAT2. After screening 153 squalamine derivatives, 7 compounds were selected for their activities on *P. aeruginosa* biofilm (formation and/or dispersion). At a concentration of 1 $\mu$ M, these compounds affected neither *P. aeruginosa* growth nor pyocyanin production, suggesting that they probably do not induce strong selection pressure, and do not affect the bacterium's virulence. By observation under CLSM and COMSTAT2 analyses, we identified a compound that induced dispersion of a preformed biofilm by 78%. Less time consuming and less expensive to synthesize than squalamine, this compound seems to be good candidate to struggle against *P. aeruginosa* biofilm. RT-qPCR analyses are also underway to identify the mechanisms involved in *P. aeruginosa* biofilm dispersion.

### **Keywords:**

*Pseudomonas aeruginosa*, biofilm, dispersion, squalamine

## Refractory *Pseudomonas aeruginosa* infection in the external auditory canal - a case report

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*Pseudomonas aeruginosa* is a well-known biofilm former. In case the biofilm formed, it becomes highly tolerant for antibiotics and immune responses. Therefore, biofilm infection is a clinical course characterized by chronic infection with intermittent acute attacks and the bacterial strains detected from each episode are identical. Patients susceptible for *P. aeruginosa* infections are usually those with immunocompromised diseases. Here we report a case of chronic *P. aeruginosa* external ear canal infection with many acute episodes in one year. Repeated conventional antibiotic treatments could not remove the infection, instead, the bacteria became more and more resistant, which become a serious challenge clinically. However, combination antibiotic treatment and application of quorum sensing inhibitor helped us successfully cure the biofilm infection. We would like to share our therapeutic experiences and lessons learned with our colleagues.

### **Keywords:**

*P. aeruginosa*, antibiotic combination, quorum-sensing

## Scratching the surface: solidifying substrates used for *Staphylococcus aureus* biofilm cultivation impact extracellular matrix composition and antibiotic tolerance

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### Background

*Staphylococcus aureus* is an opportunistic pathogen that causes a 10-30% mortality in patients with bacteremia. Further complicating *S. aureus* treatment is its ability to form biofilms, which are thought to underlie the majority of recurrent infections. Biofilms are known to enrich for persister cells, which are cells in a clonal population that can tolerate antibiotic exposure and resume growth after treatment terminates. Persistence is known to contribute to relapsing infection and poor patient outcomes. Changes in nutrient signals has been shown to be a trigger for persister formation and regrowth. Here, we investigate how nutrients on semi-solid media impact statically cultured *S. aureus* biofilms in EPS composition and antibiotic tolerance.

### Aim

Colony biofilm models often utilize agar or agarose solidifying substrates. We ask whether the solid substrate used to cultivate these biofilms impact biofilm composition, bacterial metabolism, and antibiotic persistence.

### Methods

We grew colony biofilms with a chemically defined media using either agar or agarose as the substrate. We quantify persisters that survive treatment with a new fluoroquinolone, Delafloxacin. We also measure extracellular matrix composition and bacterial metabolism in biofilms grown on each substrate.

### Results

In general, we found that biofilms grown on agar had a three-log higher survival following Delafloxacin treatment compared with those grown on an agarose substrate. We have seen these differences in multiple *S. aureus* strains and different classes of antibiotics. We found that growth on agar produced biofilms with a higher protein content compared with biofilms grown on the agarose, and this difference may contribute to antibiotic tolerance.

### Conclusions

We will further interrogate how metabolites from agar-based surfaces affect extracellular protein production in biofilms, leading to antibiotic tolerance.

We will use this information to guide our design of a colony biofilm model utilizing a nutrient composition that mimics the airway. This will allow us to confidently investigate biofilm physiology and antibiotic persistence of *S. aureus* cultured from bronchiectasis isolates from patients at UConn Health.

### Keywords:

*Staphylococcus aureus*, antibiotic persistence, nutrients

## Moringa extract promises hope in fight Staphylococcus aureus Biofilm-Associated Infections in Cystic Fibrosis Environment

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### Background

Cystic fibrosis (CF) causes mucus accumulation in the airways, creating a favourable environment for the development of chronic infections. *Staphylococcus aureus*, commonly detected in children, persists in CF lungs mainly due to biofilm formation that protects bacteria against host defences and antibiotics. Although antibiotics are ineffective against biofilms, they are the unique resource for controlling bacterial infections. Previously, we reported antibiofilm activity of moringa seeds extract against *S. aureus* surface-adhered biofilms possibly by inhibition of EPS production and/or quorum sensing. The goal of this work was to investigate the antibiofilm activity of moringa extract against polymicrobial suspended biofilms formed within CF mucus and gain insights of its mechanism of action and *S. aureus* stress response.

### Materials

Aqueous extract of moringa seeds was obtained by solid-liquid extraction with 5% solids, 1h in a water bath at 70°C and 150 rpm. Extracts were tested on in vitro CF suspended biofilms of *S. aureus* and in mixed biofilms of *P. aeruginosa* formed in artificial CF sputum media (ASM) to mimic in vivo conditions.

### Results

Results confirmed the antibiofilm activity of moringa extract since it inhibited *S. aureus* growth and biofilm formation in ASM for 5 days with a single application. In polymicrobial biofilms, *P. aeruginosa* provided protection to *S. aureus*. Moringa extract did not inhibit *S. aureus* growth but induced a slow growth rate as colonies on solid medium were only detected after 3 days of growth. Two new colony morphotypes were detected including a small colony variant. *S. aureus* response to moringa extract was adaptive or transient. After removal of moringa from ASM, bacterial growth kinetics and biofilm formation was identical to the control group. Moringa did not trigger increased tolerance to antibiotics.

### Conclusion

Data showed that moringa extract inhibits *S. aureus* biofilm formation and induces slow growth in polymicrobial biofilms. Moringa extract can be a promising anti-staphylococcal drug candidate and may prevent the development of biofilm-associated chronic infections in CF environment.

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### Keywords:

Antibiofilm, Plant extracts, *Staphylococcus aureus*

## Polymicrobial biofilms shorten the selection of novel antibiofilm therapies for cystic fibrosis-associated infections

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*Pseudomonas aeruginosa* is the main responsible for cystic fibrosis (CF) chronic respiratory infections that are though to treat. Antibiotics remain the centerpiece of CF infections management but the correlation between antibiotherapy and clinical outcome is still poor, mostly because of the presence of biofilms, settling down the need for seeking therapies targeting biofilms. This study aimed challenging the antibiofilm activity of aspartic acid (ASP) and its adjuvant effect on tobramycin (TOB) against dual-species biofilms formed in an in vitro model resembling the CF airway environment. The prophylactic antibiofilm effect of ASP-TOB co-application were assessed in dual-species biofilms, developed on artificial CF sputum medium after 24 h of growth, by CFU counting. The biofilms were formed combining *P. aeruginosa* with *Staphylococcus aureus* and *Burkholderia cenocepacia*. Furthermore, to gain insights on ASP mode of action, its antibacterial effects were evaluated after 24 h by characterizing the planktonic bacterial growth kinetics (OD measuring) and biofilm formation (CFU counting; biomass quantification; structure inspection) in tryptic soy broth. Data showed that ASP-TOB co-administration triggers a synergistic action on single *P. aeruginosa* biofilms since its antibacterial features exceeded the added effect of the two drugs individually. This brings to light ASP antibiofilm activity. Indeed, biofilm biomass and CLSM results showed that ASP efficiently inhibited biofilm formation. ASP mechanism of action is possibly related to EPS production and/or quorum sensing inhibition because cells were adhered to surface, but no matrix production was observed. However, the susceptibility of *P. aeruginosa* to ASP-TOB treatment decreased when entailed in dual-species biofilms, highlighting that interspecies interactions have a key role in the response of the consortia towards antimicrobial therapies. This study strengthens ASP as having promising antibiofilm activity and its delivery to CF airway environment seemed to improve tobramycin efficacy overcoming *P. aeruginosa* insusceptibility and to inhibit single-species biofilm formation. Yet, the presence of non-pseudomonal species within the biofilms interferes with therapy efficiency. Acknowledgements : Portuguese Foundation for Science and Technology(FCT) through the strategic funding of UIDB/04469/2020 unit (DOI 10.54499/UIDB/04469/2020) and by LABELS, LA/P/0029/2020; FCT for grants of RM (2020.04988.BD) and for AMS Scientific Employment Stimulus (<https://doi.org/10.54499/CEECIND/01507/2017/CP1458/CT0005>).

### Keywords:

Biofilm-associated infections, *Pseudomonas aeruginosa*, antibiofilm

## Drug conjugates for targeted delivery of antineoplastic drugs to biofilm infections

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One of the key mechanisms for biofilm infections to become chronic is that non-growing persister cells in the biofilm do not respond to conventional antibiotics. However, some antineoplastic drugs used in cancer treatment have antimicrobial activity and are equally effective against persister cells and actively growing cells. The only problem is that these drugs are cytotoxic and immunosuppressive. So, can they be used to treat a biofilm infection?

Our goal is to deliver the antineoplastic drug mitomycin C to biofilms in the form of an inactive prodrug that is only activated at the site of infection. The idea is that such targeted delivery will minimize off-target activity and thereby side effects.

We developed an antibody-drug conjugate in which mitomycin was attached to a *Staphylococcus aureus*-specific antibody via a biodegradable linker that breaks upon interaction with reactive thiols. Such thiols are present on the surface of bacteria, and we showed a targeted antimicrobial activity of the antibody-drug conjugate in vitro and in vivo.

Now we redesign this concept by using the same linker chemistry to conjugate mitomycin to a small molecule targeting agent with broad-spectrum binding affinity for Gram positive bacteria: Vancomycin. After identifying the optimal linker position, we show that the vancomycin- mitomycin conjugates also deliver targeted antimicrobial activity against Gram positive pathogens.

### **Keywords:**

Drug conjugates, antineoplastic, antibiotic